Calcium uptake and distribution in relation to periods of active white root growth in young, potted apple trees (*Malus domestica* Borkh. cv. 'Golden Delicious') in the Western Cape

by

Anouska Cameron

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of AgriSciences at Stellenbosch University

Supervisor:	Dr. E. Lötze	Department of Horticultural Science Stellenbosch University
Co-supervisor:	Dr. L. Hoffman	Department of Horticultural Science Stellenbosch University

April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature: _____ Date: _____

SUMMARY

Two-year-old 'Golden Delicious'/M7 apple trees were chosen as the experimental material for this study. Potted trees were fertigated with a low calcium (Ca), balanced nutrient solution over two consecutive seasons (2015/16 and 2016/17). In the first season, trees were either left to drop their leaves naturally (NLD) during autumn and winter or were completely defoliated by hand earlier in autumn (AD), during April 2016. At the beginning of the second root flush, in winter (May 2016), calcium nitrate ($Ca(NO_3)_2$) was applied to the soil at three different rates: no additional Ca (control), moderate Ca (1X) and high Ca (2X). At the end of winter (August 2016), all trees were destructively sampled for macro mineral analysis of the different plant organs.

Although an increase in soil Ca supply did not have a significant impact on white root numbers during winter (determined by minirhizotrons), early leaf loss via AD caused a drastic decline in white root numbers during winter. Despite this, Ca uptake and reserve accumulation in the roots and stems of the $2X_{(AD)}$ treatment was not compromised, indicating possible active uptake by older, brown roots. Sap flow rates in leafless trees (determined by dendrometers) also remained above zero during winter, suggesting that leaf transpiration-driven sap flow is not essential for root Ca uptake and translocation in the xylem. In the NLD trees, an increase in autumn soil Ca supply resulted in a significant increase in leaf Ca concentration following the extended leaf drop period. At the same time, scanning electron microscopy and wavelength-dispersive x-ray spectroscopy (SEM-WDS) results indicated rapid uptake of soil-applied Ca by apple white root tips during winter. As 50 % leaf drop was reached late in winter (July), a substantial fraction of total Ca uptake in the $2X_{(NLD)}$ treatment was lost via leaf abscission at the expense of Ca allocation to the reserve tissues during winter. The preferential allocation of Ca to the leaves was possibly the result of relative high rates of leaf transpiration-driven sap flow prior to 50 % leaf drop.

Calcium partitioning in relation to periods of active white root growth, after harvest in the second season, were also investigated. Treatments comprising $Ca(NO_3)_2$ soil applications included a summer-only (2X) treatment, an autumn-only (2X) treatment and a summer (1X)/autumn (1X) treatment. Although fruit Ca concentrations were satisfactory at harvest, no significant differences were found between treatments. Whilst confirming primary xylem transport to the more dominant leaf and shoot sinks, the lack of response to summer

applications (current season, 2016/17) indicates the predominant role of Ca reserve accumulation in the trees in supporting new growth the following season. In contrast to the summer/autumn treatment, a significantly higher % of total Ca content was found in the fruit of the autumn-only treatment. These results suggest that under local conditions of insufficient winter chilling, relatively high rates of soil-applied $Ca(NO_3)_2$, both in summer and autumn during active white root growth, may benefit the next season's crop through remobilization of stored Ca in the roots and reserve tissues of the stems.

OPSOMMING

Kalsium opname en verspreiding teenoor periodes van aktiewe witwortelgroei in jong appel bome (*Malus domestica* Borkh. kv. 'Golden Delicious') in potte in die Weskaap

Twee-jaar 'Golden Delicious'/M7 appelbome is as eksperimentele materiaal vir hierdie studie gekies. Bome is uitgeplant in potte en sproeibemes met 'n lae Ca, gebalanseerde voedingsoplossing oor twee seisoene (2015/16 en 2016/17). In die eerste seisoen, is bome of gelaat om normale blaarval (NBV) te ondergaan gedurende die herfs en winter, of per hand ontblaar in die herfs (HO), gedurende April 2016. Aan die begin van die tweede witwortel groeifase, in die winter (Mei 2016), is bome behandel met drie verskillende konsentrasies van kalsiumnitraat ($Ca(NO_3)_2$) as grondtoedienings: geen addisionele Ca (kontrole), matige Ca (1X) en hoë Ca (2X). Aan die einde van die winter (Augustus 2016), is alle bome destruktief gemonster vir minerale (makro) analises van die verskillende plant organe.

Al het 'n toename in grond Ca toevoer nie 'n betekenisvolle impak op witwortel getalle in die winter gehad nie (bepaal deur minirhizotrons), het vroeë blaarverlies deur HO tot 'n drastiese afname in witwortel getalle gedurende die winter gelei. Ten spyte daarvan, is Ca opname en reserwe akkumulasie in die wortels en stamme van die $2X_{(HO)}$ behandeling nie negatief geaffekteer nie, wat dui op moontlike aktiewe opname deur ouer, bruin wortels. Sapvloeitempo in blaarlose bome (bepaal deur dendrometers) het ook bokant nul gebly gedurende die winter, wat daarop dui dat blaar transpirasie-gedrewe sapvloei nie noodsaaklik is vir Ca opname en translokasie in die xileem nie. Na die verlengde blaarval tydperk in die NBV bome, het 'n toename in grond Ca toevoer tot 'n beduidende toename in blaar Ca konsentrasie gelei. Terselfdertyd, dui skanderingselektromikroskopie en golflengte-dispersiewe x-straal spektroskopie (SEM-WDS) resultate op vinnige opname van grondtoedienings van Ca in appel witwortelpunte gedurende die winter. Aangesien 50 % blaarval eers laat in die winter (Julie 2016) bereik is, was 'n aansienlike fraksie van die totale Ca opname in die $2X_{(NBV)}$ behandeling deur middel van blaarval verloor, ten koste van Ca allokasie na die reserwe weefsels gedurende die winter. Die hoë persentasie Ca wat na die blare geallokeer is, is die moontlike gevolg van relatiewe hoë blaar transpirasie-gedrewe sapvloeitempo's in die bome voor 50 % blaarval.

Die verwantskap tussen Ca opname en verspreiding teenoor periodes van aktiewe witwortelgroei na oes, in die tweede seisoen, is ook ondersoek. Behandelings bestaande uit $Ca(NO_3)_2$ grondtoedienings, het ‘n somer-alleen (2X), ‘n herfs-alleen (2X) en ‘n somer (1X)/herfs (1X) behandeling ingesluit. Vrug Ca konsentrasies tydens oes was bevredigend en daar was geen betekenisvolle verskille in vrug Ca konsentrasie tussen behandelings nie. Terwyl primêre xileem vervoer na die meer dominante blaar- en lootsinke bevestig is, dui die gebrek aan reaksie op somer grondtoedienings (huidige seisoen, 2016/17) op die oorheersende rol van Ca opberging in die bome om nuwe groei in die volgende seisoen te ondersteun. In teenstelling met die somer/herfs behandeling, is ‘n noemenswaardige hoër % van totale Ca inhoud in die vrugte van die herfs-alleen behandeling gevind. Hierdie resultate dui daarop dat onder plaaslike toestande van onvoldoende winterkoue, mag relatiewe hoë vlakke van $Ca(NO_3)_2$ grondtoedienings, beide in die somer en die herfs tydens aktiewe witwortelgroei, moonlik die daaropvolgende seisoen se oes bevoordeel deur remobilisasie van opgebergte Ca in die wortels en reserwe weefsels in die stamme.

DEDICATION

Dedicated to my beloved parents Frans and Narina de Klerk

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude towards the following people and institutions:

- My supervisor, Dr. E. Lötze, for her guidance, tireless patience, encouragement and excellent advice during my studies.

- My co-supervisor, Dr. L. Hoffman, for her brilliant insights and support throughout this project.

- Mr. G. Lötze and his staff at the Department of Horticultural Science, Stellenbosch University, with special mention to Mr. T. Groenewald and Mr. A. Swartz, for their assistance in the field and laboratory.

- Mr. W. van Kerwel and his staff at the Welgevallen Experimental Farm for their assistance during spraying and harvesting of the trees.

- Yara Western Cape for their valuable contributions and funding of this project, with special mention to Mr. P. van der Merwe.

- My colleagues in the Top of the Lötze group and Mrs. S. Horn, for their friendship, assistance and encouragement during my studies.

- My family, friends and fiancé Cobus Beukes for their boundless love, loyal support and motivation throughout my studies.

This thesis is a compilation of chapters, starting with a literature review, followed by three research papers. Each paper is an individual entity and as thus, some repetition or duplication between papers has been unavoidable.

TABLE OF CONTENTS

GENERAL INTRODUCTION

Calcium (Ca) is an essential macro nutrient that plays an important role in many functions related to the physiology and structural processes within trees (Conn and Gilliham, 2010; Lautner and Fromm, 2010; Hocking et al., 2016). As a divalent cation (Ca^{2+}), it acts as a second messenger in the cytosol, maintains the charge or osmotic balance within cells by acting as a counter-cation for inorganic and organic anions in the vacuole, maintains the structural integrity of cells through its decisive role in cell wall strengthening and membrane stabilization and is required for cell division and the proper growth of tree organs (Kirkby and Pilbeam, 1984; White and Broadley, 2003; Hirschi, 2004; McAinsh and Pittman, 2009; Hawkesford et al., 2012). Calcium deficiency symptoms in apple fruit are seldom noticed in the field, but rather appear as physiological disorders postharvest (Millaway and Wiersholm, 1979; Ferguson et al., 1999; Hewett, 2006). To reduce or prevent the risk of large economic losses caused by these disorders, programs that include numerous exogenous applications of calcium nitrate ($Ca(NO_3)_2$), calcium chloride ($CaCl_2$) or organic salt formulations that contain Ca, sprayed directly onto the fruit prior to harvest, are widely recommended as a routine complementary source of Ca to raise fruit Ca levels above the threshold limit (Lötze and Theron, 2006, 2007; Lötze et al., 2008; Val et al., 2008; Blanco et al., 2010; Casero et al., 2010; Torres et al., 2017). However, as it has been indicated that in-season spraying often delivers inconsistent results in increasing fruit Ca levels at harvest (Lötze and Theron, 2006, 2007; Lötze et al., 2008), the importance of adequate soil Ca supply should not be underestimated.

Direct effects of Ca deficiency on 'Golden Delicious' apple fruit quality, including poor storage quality and the development of physiological disorders such as bitter pit (Khan et al., 2006; Lötze and Theron, 2006, 2007; Lötze et al., 2008) depends on a range of preharvest factors that control the uptake and translocation of Ca^{2+} to the fruit (Ferguson et al., 1999; Saure, 2005; Napier and Combrink, 2006; De Freitas and Mitcham, 2012; Jemrić et al., 2016). Some of these factors include the relative content and availability of Ca^{2+} and other nutrients e.g. potassium (K^+), ammonium (NH_4^+), magnesium (Mg^{2+}) and nitrate (NO_3^-) in the soil/fertigation solution, root zone temperature and soil moisture level, the presence of young white roots or root tips and leaf: fruit ratio which, depending on their relative rates of transpiration and growth, could limit Ca^{2+} movement to rapidly developing fruit. It is generally accepted that apple fruit quality during storage is favoured by high Ca: N, Mg and K ratios in the fruit (Raese and Staiff, 1990; Amarante et al., 2006a, b, 2013; Casero et al., 2010; De Freitas et al., 2010, 2015; Miqueloto

et al., 2014). Due to its low mobility in the phloem, Ca uptake and translocation proceeds upwards through the xylem, after absorption by new roots, and is dependent on the solution concentration, the rate of transpiration and/or growth-related demand (Hanger, 1979; Clarkson, 1984; Engels, 1999; White and Broadley, 2003; Gilliham et al., 2011). Furthermore, the total amount of Ca in apple fruit at harvest accounts for both Ca taken up by the roots in the current season and cumulative Ca recycled from previous seasons (Terblanche, 1972; Terblanche et al., 1979; Ferguson, 1980; Wilsdorf, 2011). The extent to which Ca located in the permanent organs such as the roots and woody tissues of the stems can contribute to supply, is particularly important during the early stages of fruit development when functional xylem is still the main pathway of Ca movement to the fruit (Dražeta et al., 2001, 2004; Miqueloto et al., 2014; Le Roux, 2018) and direct supply of soil-applied Ca from the roots is still inadequate (Kangueehi, 2008; Wilsdorf, 2011; Van Zyl, 2016). Thus, to manipulate fruit Ca levels in locally grown apple trees through optimal timing of soil Ca supply, it is of utmost importance to know the seasonal dynamics of Ca uptake and distribution in relation to periods of active white root growth in these trees.

The aim of this study was to quantify Ca uptake and distribution in relation to periods of active white root growth in young, potted apple trees (*Malus domestica* Borkh. cv. 'Golden Delicious') in Stellenbosch (Western Cape), a region in South Africa known to experience insufficient winter chilling (Midgley and Lötze, 2011). Due to widespread evidence that young white roots or root tips have the highest potential for soil nutrient uptake (Clarkson, 1984, 1993; Bouma et al., 2001; Volder et al., 2005; De Freitas and Mitcham, 2012; Gu et al., 2015), we began the study with a literature review, highlighting existing information on the link between fine root function and root trait variation among individual roots within the branched hierarchy that differ in development (fibrous vs. pioneer), age and lifespan, focusing on root Ca uptake in apple.

To confirm existing findings regarding considerable root growth during summer (first white root flush) and autumn/winter (second white root flush) (Van Zyl, 2016), minirhizotrons were used for in situ monitoring and quantification of root growth dynamics over two seasons in relation to above-ground tree phenology (Hendrick and Pregitzer, 1996; Johnson et al., 2001; Gluszek et al., 2013; Judd et al., 2015).

Three trials were conducted. In the first trial, we investigated whether there is active Ca uptake in apple trees during autumn and into winter. Although active uptake following $Ca(NO_3)_2$ soil applications in autumn was indicated by Van Zyl (2016), the question pertaining to how high concentrations of Ca in the roots and stems are attained in the absence of leaf transpiration-driven sap flow in the xylem, was not addressed. An investigation followed to determine whether there is considerable Ca uptake during the second root flush in the season, without and with leaf removal, to establish the impact of transpiration and leaf drop on Ca reserve accumulation in apple trees at the end of winter. In addition, the effect of various levels of soil $Ca(NO_3)_2$ supply during autumn and into winter on root growth and Ca partitioning in apple trees was evaluated.

In the second trial, scanning electron microscopy (SEM) and wavelength-dispersive x-ray spectroscopy (WDS) was used to determine the impact of soil Ca supply during the second root flush on Ca uptake and distribution along the length of apple white root tips in relation to above-ground tree phenology. To our knowledge, this has not yet been investigated or quantified. Reportedly, SEM coupled with x-ray microanalysis, i.e. WDS or energy-dispersive x-ray spectroscopy (EDS), is a reliable technique to study nutrient/element localization and quantification in plant tissues (Storey and Leigh, 2004; Cocozza et al., 2008; Hunsche and Noga, 2008; Akhter et al., 2014).

In the third trial, we aspired to determine the optimal timing of soil Ca supply to enhance the Ca content in ‘Golden Delicious’ apple fruit at harvest. The main objective was to determine if Ca uptake and partitioning to the fruit can be increased substantially by applying additional $Ca(NO_3)_2$ to the soil during the first root flush, during the second root flush, or both during the first root flush and the second root flush of the season. We hypothesised that active Ca uptake during winter contributes towards fruit Ca levels of the following season’s fruit via focussed allocation of additional soil-derived Ca towards the storage tissues of the above-ground tree organs as well as the roots. According to Terblanche et al. (1979), early-season remobilization of readily exchangeable Ca from the roots and reserve tissues (wood and bark) can contribute up to 25 % of the total Ca content in the new growth (leaves, shoots and fruit) in spring. Compared to summer and autumn/winter, new root growth in locally-grown apple trees is negligible during spring (Van Zyl, 2016). It is thus imperative to target fruit early in the season via remobilization of stored reserves from the roots and stems following soil Ca uptake, as this is their main source of Ca supply.

References

Akhter, M.F., Omelon, C.R., Gordon, R.A., Moser, D., Macfie, S.M., 2014. Localization and chemical speciation of cadmium in the roots of barley and lettuce. Environ. Exp. Bot. 100, 10–19.

Amarante, C.V.T., Chaves, D.V., Ernani, P.R., 2006a. Mineral composition and bitter pit severity in 'Catarina' apples. Rev. Bras. Frutic. 28, 51–54.

Amarante, C.V.T., Chaves, D.V., Ernani, P.R., 2006b. Multivariate analysis of nutritional attributes associated with bitter pit in 'Gala' apples. Pesq. Agropec. Bras. 41, 841–846.

Amarante, C.V.T., Miqueloto, A., Steffens, C.A., dos Santos, A., Argenta, L.C., 2013. Changes in xylem functionality during apple fruit development: Implications on calcium concentration and incidence of bitter pit. Acta Hort. 1012, 135–140.

Blanco, A., Fernández, V., Val, J., 2010. Improving the performance of calcium-containing spray formulations to limit the incidence of bitter pit in apple (*Malus* × *domestica* Borkh.) Sci. Hort. 127, 23–28.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Casero, T., Benavides, A.L., Recasens, I., 2010. Interrelation between fruit mineral content and pre-harvest calcium treatments on 'Golden Smoothee' apple quality. J. Plant Nutr. 33, 27–37.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

Cocozza, C., Minnocci, A., Tognetti, R., Iori, V., Zacchini, M., Scarascia Mugnozza, G., 2008. Distribution and concentration of cadmium in root tissue of *Populus alba* determined by scanning electron microscopy and energy-dispersive x-ray microanalysis. IForest 1, 96–103.

Conn, S., Gilliham, M., 2010. Comparative physiology of elemental distributions in plants. Ann. Bot. 105, 1081–1102.

De Freitas, S.T., Amarante, C.V.T., Labavitch, J.M., Mitcham, E.J., 2010. Cellular approach to understand bitter pit development in apple fruit. Postharvest Biol. Technol. 57, 6–13.

De Freitas, S.T., Amarante, C.V.T., Mitcham, E.J., 2015. Mechanisms regulating apple cultivar susceptibility to bitter pit. Sci. Hort. 186, 54–60.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hort. Rev. 40, 107–146.

Dražeta, L., Lang, A., Hall, A.J., Volz, R.K., Jameson, P.E., 2004. Causes and effects of changes in xylem functionality in apple fruit. Ann. Bot. 93, 375–382.

Dražeta, L., Lang, A., Morgan, L., Volz, R., Jameson, P.E., 2001. Bitter pit and vascular function in apples. Acta Hort. 564, 387–392.

Engels, C., 1999. Regulation of xylem transport of calcium from roots to shoot of maize by growth-related demand. J. Plant Nutr. Soil Sci. 162, 287–294.

Ferguson, I.B., 1980. The uptake and transport of calcium in the fruit tree, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 183–192.

Ferguson, I., Volz, R., Woolf, A., 1999. Preharvest factors affecting physiological disorders of fruit. Postharvest Biol. Technol. 15, 255–262.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Gluszek, S., Paszt, L.S., Sumorok, B., Derkowska, E., Kozera, R., 2013. Application of the minirhizotron technique to studying the roots of fruit plants. Adv. Sci. Technol. Res. J. 7, 45–53.

Gu, J., Wei, X., Wang, J., Dong, X., Wang, Z., 2015. Marked differences in standing biomass, length density, anatomy and physiological activity between white and brown roots in *Fraxinus mandshurica* Rupr. plantation. Plant Soil 392, 267–277.

Hanger, B.C., 1979. The movement of calcium in plants. Commun. Soil Sci. Plant Anal. 10, 171–193.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Moller, I.S., White, P., 2012. Functions of macronutrients, Marschner's Mineral Nutrition of Higher Plants.

doi:10.1016/B978-0-12-384905-2.00006-6.

Hendrick, R.L., Pregitzer, K.S., 1996. Applications of minirhizotrons to understand root function in forests and other natural ecosystems. Plant Soil 185, 293–304.

Hewett, E.W., 2006. An overview of preharvest factors influencing postharvest quality of horticultural products. Int. J. Postharvest Technol. Innov. 1, 4–15.

Hirschi, K.D., 2004. The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol. 136, 2438–2442.

Hocking, B., Tyerman, S.D., Burton, R.A., Gilliham, M., 2016. Fruit Calcium: Transport and Physiology. Front. Plant Sci. 7, 1–17.

Hunsche, M., Noga, G., 2008. Applicability of the energy dispersive x-ray microanalysis for quantification of irregular calcium deposits on fruit and leaf cuticles. J. Microsc. 232, 453–462.

Jemrić, T., Fruk, I., Fruk, M., Radman, S., Sinkovič, L., Fruk, G., 2016. Bitter pit in apples: Pre- and postharvest factors: A review. Spanish J. Agric. Res. 14, 1–12.

Johnson, M.G., Tingey, D.T., Phillips, D.L., Storm, M.J., 2001. Advancing fine root research with minirhizotrons. Environ. Exp. Bot. 45, 263–289.

Judd, L., Jackson, B., Fonteno, W., 2015. Advancements in root growth measurement technologies and observation capabilities for container-grown plants. Plants 4, 369–392.

Kangueehi, G.N., 2008. Nutrient requirement and distribution of intensively grown 'Brookfield Gala' apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Khan, F.A., Rather, A.H., Qazi, N.A., Bhat, M.Y., Darzi, M.S., Beigh, M.A., Ahmad, I., 2006. Effect of modified atmosphere packaging on maintenance of quality in apple. J. Hort. Sci. 1, 135–137.

Kirkby, E.A., Pilbeam, D.J., 1984. Calcium as a plant nutrient. Plant Cell Environ. 7, 397–405.

Lautner, S., Fromm, J., 2010. Calcium-dependent physiological processes in trees. Plant Biol. 12, 268–274.

Le Roux, E., 2018. Ca-metalosate as an alternative Ca formulation for decreasing Ca related disorders in fruit trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Lötze, E., Joubert, J., Theron, K.I., 2008. Evaluating pre-harvest foliar calcium applications to

increase fruit calcium and reduce bitter pit in 'Golden Delicious' apples. Sci. Hort. 116, 229–304.

Lötze, E., Theron, K.I., 2006. Dynamics of calcium uptake with pre-harvest sprays to reduce bitter pit in 'Golden Delicious'. Acta Hort. 721, 313–319.

Lötze, E., Theron, K.I., 2007. Evaluating the effectiveness of pre-harvest calcium applications for bitter pit control in 'Golden Delicious' apples under South African conditions. J. Plant Nutr. 30, 471–485.

McAinsh, M.R., Pittman, J.K., 2009. Shaping the calcium signature. New Phytol. 181, 275–294.

Midgley, S.J.E., Lötze, E., 2011. Climate change in the Western Cape of South Africa: Trends, projections and implications for chill unit accumulation. Acta Hort. 903, 1127–1134.

Millaway, R.M., Wiersholm, L., 1979. Calcium and metabolic disorders. Commun. Soil Sci. Plant Anal. 10, 1–28.

Miqueloto, A., Amarante, C.V.T., Steffens, C.A., dos Santos, A., Mitcham, E., 2014. Relationship between xylem functionality, calcium content and the incidence of bitter pit in apple fruit. Sci. Hort. 165, 319–323.

Napier, D.R., Combrink, N.J., 2006. Aspects of calcium nutrition to limit plant physiological disorders. Acta Hort. 702, 107–116.

Raese, J.T., Staiff, D.C., 1990. Fruit calcium, quality and disorders of apples (*Malus domestica*) and pears (*Pyrus communis*) influenced by fertilizers, in: Van Beusichem, M.L. (Ed.), Plant Nutrition - Physiology and Applications. Kluwer Academic Publishers, Dordrecht, pp. 619–623.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: Its mechanism and endogenous control. Sci. Hort. 105, 65–89.

Storey, R., Leigh, R.A., 2004. Processes modulating calcium distribution in citrus leaves. An investigation using x-ray microanalysis with strontium as a tracer. Plant Physiol. 136, 3838–3848.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Torres, E., Recasens, I., Lordan, J., Alegre, S., 2017. Combination of strategies to supply calcium and reduce bitter pit in 'Golden Delicious' apples. Sci. Hort. 217, 179–188.

Val, J., Monge, E., Risco, D., Blanco, A., 2008. Effect of pre-harvest calcium sprays on calcium concentrations in the skin and flesh of apples. J. Plant Nutr. 31, 1889–1905.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Volder, A., Smart, D.R., Bloom, A.J., Eissenstat, D.M., 2005. Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: Implications for root efficiency and competitive effectiveness. New Phytol. 165, 493–501.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilsdorf, R., 2011. Evaluating the seasonal changes in calcium concentration and distribution in apple fruit after application of different calcium fertilisation strategies. MSc thesis, Faculty of Agrisciences, Stellebosch University.

LITERATURE REVIEW

Exploring the link between fine root function and root trait variation for calcium uptake in apple trees

1. Introduction

The root system of perennial trees, including apple, consists of a structural framework of long woody roots that radiate both horizontally and vertically through the soil volume, as well as a network of lateral fine roots that exploit the upper soil volume, arising either directly from the framework roots, or indirectly as higher order branches (Atkinson and Wilson, 1980; McCully, 1999; Fitter, 2002; Pregitzer, 2002). A substantial volume of soil can be colonized by the roots which may vary from 2 m^3 for a one-year-old tree to over 100 m^3 for a four-year-old tree, as has been shown for plums (Vercambre et al., 2003). Compared to citrus and other deciduous fruit crops, apple trees have a relatively sparse root system (Atkinson, 1983; Hughes and Gandar, 1993; Bouma et al., 2001; Eissenstat et al., 2001) with its structure and spatial distribution dependent on various factors including: the rootstock/interstock/scion combination; planting density; soil type and soil management practices; irrigation; fertilization and cultural practices; tree age, year and season (De Silva et al., 1999; Bianco et al., 2003; Eissenstat et al., 2006; Sokalska et al., 2009; Yao et al., 2009; Fan and Yang, 2011; Hu et al., 2011; Ma et al., 2013; Polverigiani et al., 2013; An et al., 2017). Despite their relatively low root length densities, apple trees are able to produce remarkably high yields compared to other deciduous fruit crops (Eissenstat et al., 2001), presumably as a result of their high root efficiency, defined as the ratio of nutrients (or water) gained (= benefit) per unit carbon (C) expended in respiration (= cost) for root construction, growth, ion uptake and maintenance (Eissenstat and Yanai, 1997; Eissenstat et al., 2001; Eissenstat and Volder, 2005).

Comprising most of the total root length and occupying the greatest volume of soil per unit root volume, fine roots represent the largest surface area of the tree root system (Atkinson, 1983; Pregitzer, 2002; Guo et al., 2004; Pierret et al., 2005; Rewald et al., 2011). In apple, the majority (> 70 %) of fine roots are most often located within 0.4 m of the soil surface under uniform wet conditions (Atkinson, 1974; Green and Clothier, 1999). Although fine roots, with or without mycorrhizal associates, primarily function in soil resource acquisition, they also

play a key role in the synthesis of plant growth regulators (e.g. cytokinin) and in ecosystem C and nitrogen (N) cycling via rapid turnover and decomposition. Contingent on their type and/or branching position, fine roots also serve functions of transport, storage and anchorage via lateral root production (Fitter, 2002; Pregitzer, 2002; Zadworny and Eissenstat, 2011; Bagniewska-Zadworna et al., 2012, Bardgett et al., 2014; McCormack et al., 2015).

Fine roots have traditionally been classified according to arbitrary diameter-based cut-offs. Depending on the species, this system designates all roots < 1 or 2 mm in diameter as "fine roots", and all roots > 2 mm in diameter as "coarse roots". Treated as one coherent mass, all fine roots were assumed to be short-lived, structurally identical and functionally similar (i.e. absorptive rather than conductive) without taking into consideration possible differences in their morphology, anatomy and physiology (Pregitzer, 2002; McCormack et al., 2015; Weemstra et al., 2016). However, it is now widely recognized that resource absorption is not constant along the total fine root length of trees (Valenzuela-Estrada et al., 2008; Xia et al., 2010; Rewald et al., 2014; McCormack et al., 2015). In fact, it has been shown that the single diameter class approach could overestimate fine root absorptive length in trees by 25 %, as some fine roots may have their primary function in transport and storage rather than absorption (Guo et al., 2008a). Fine root absorptive length may also vary considerably through the growing season, as estimates of active absorption within the fine-root biomass between 1 – 85 % have been reported for apple (Mackenzie, 1979) and between 10 – 58 % or higher (> 75 %) for various other tree species (Guo et al., 2008a; McCormack et al., 2015).

While it is widely accepted that young white roots or root tips have the highest potential for soil nutrient uptake (Clarkson, 1984, 1993; Bouma et al., 2001; Volder et al., 2005; De Freitas and Mitcham, 2012; Gu et al., 2015), root browning with age may not be indicative of a total loss of functionality. Root absorption capacity, however, depends on the root type, the developmental state of the root and the specific nutrient element being absorbed (Comerford et al., 1994; Mckenzie and Peterson, 1995a, b; Escamilla and Comerford, 2000; Wells and Eissenstat, 2003). For calcium (Ca) uptake in particular, differences in the degree of absorption along the length of individual roots are well known to be a direct consequence of root structural development with age (Clarkson, 1984; White, 2001; White and Broadley, 2003). Furthermore, numerous studies over the last few decades have shown that a shift in fine root function from resource absorption to transport and/or storage is linked to marked variations in key root functional traits, which include the morphology, anatomy, chemistry, physiology and

mycorrhizal associations among individual roots within the fine root architecture, both spatially, i.e. within and between branching orders on the same root system, and temporally, i.e. with age (Wells and Eissenstat, 2003; Hishi, 2007; McCormack et al., 2015; Lalibert, 2017).

Since a clear understanding of the link between fine root function and root trait variation is needed if we are to improve current root sampling protocols for the study of nutrient uptake dynamics in trees (Freschet and Roumet, 2017; McCormack et al., 2017), the aim of this paper will be to highlight existing knowledge on the variation in key root functional traits among individual roots within the fine root architecture that differ in development, age and lifespan, focussing on root Ca uptake in apple.

2. Seasonal pattern of fine root production in apple

Determination of root Ca uptake in apple, first requires a basic understanding of the seasonal pattern of fine root production in these trees. General evidence suggests that young, non-bearing apple trees have the potential to continuously produce new white roots at some level (with less pronounced peaks) throughout the growing season, especially in the first year after planting. For mature, bearing apple trees the tendency is more to produce large quantities of roots during one or more distinct seasonal "flushes" (Head, 1966, 1967, 1969; Cripps, 1970; Atkinson and Wilson, 1980; Atkinson, 1983; Psarras et al., 2000; Eissenstat et al., 2006; Van Zyl, 2016) where the onset and duration of each flush reflects the influence of various factors including: climate; soil type; soil temperature and moisture levels; rootstock/interstock/scion combination; above-ground pruning practices and fruit load (Head, 1967, 1969; Atkinson and Wilson, 1980; Eissenstat et al., 2006; Yao et al., 2006; Ma et al., 2013).

In the northern hemisphere, root production mostly occurs asynchronously with shoot growth (Head, 1967; Atkinson, 1983; Ma et al., 2013). An initial flush often occurs during or shortly after full bloom in spring that is either followed by a period of low to moderate root production for the rest of the growing season, or by a second strong root growth flush in late-summer/early-autumn after shoot growth has ceased or after harvest. An increase in root production may also occur from early- to mid-summer, either preceding the latter peak, or replacing it (Head, 1966, 1967; Psarras et al., 2000; Eissenstat et al., 2006; Yao et al., 2006; Ma et al., 2013). In these parts of the world, active root growth during late-autumn/winter has rarely been reported for

apple, presumably because the soils are too cold to sustain active growth (Head, 1966; Pregitzer et al., 2000; Psarras et al., 2000; Eissenstat et al., 2006).

In the southern hemisphere, particularly in apple growing regions with a Mediterranean-type climate, root production tends to follow a more pronounced bimodal pattern. Utilizing minirhizotrons to investigate white root dynamics in various apple orchards in the warmer growing regions of the Western Cape, South Africa, Van Zyl (2016) found that an initial minor flush occurs early in summer prior to the major fruit growth stage but overlapping with shoot growth (November/December), followed by a second major flush, postharvest, during autumn and winter (March to August). The second root growth peak was attributed to an extended leaf drop period in response to local growing conditions. Cripps (1970) reported similar findings for apple orchards in Western Australia. However, in contrast with those in the Western Cape, the second peak in autumn was less pronounced and of shorter duration compared with the first peak in summer, and root activity during winter was negligible. Lack of active root growth in winter was attributed to tree dormancy and the absence of actively photosynthesizing leaves. For future investigations in apple, these differences thus underscore the importance of examining the role of whole tree phenology in the regulation of seasonal fine root production.

3. Branched hierarchy of the fruit tree root system

With each seasonal root growth flush, multiple orders of lateral fine roots are produced in branches along the axes of parent roots. Two types of lateral branch roots have been identified, namely “primary branch roots” (PBR), arising from primary meristematic tissues (i.e. the pericycle) located between the phloem and endodermis in the apical non-woody zone of the parent root, and “adventitious or secondary branch roots” (SBR), arising from derivatives of the vascular cambium associated with the secondary phloem tissues in the basal woody zone of the parent root (Paolillo and Bassuk, 2005; Chiatante et al., 2007, 2010). Likewise, Head (1966) referred to new lateral root production from both thickened (secondary developed) and non-thickened (primary developed) apple roots. Although PBR and SBR are morphologically indistinct (Paolillo and Bassuk, 2005; Chiatante et al., 2007; Chiatante et al., 2010), SBR are mostly produced after periods of injury or environmental stress when root growth temporarily slows down or stops (Sutton, 1980; Pagès and Serra, 1994).

The relative position of individual roots within the branched hierarchy depicts their order (Fitter et al., 1991; Fitter, 2002). Either one of two classification schemes may be used to assign roots to a specific order. The first is based on development or ontogeny (classical approach), and the second, on branch structure or topology (Hodge et al., 2009). In the first scheme (Fig. 1A), root order is assigned according to the sequence of lateral root emergence. Here, lateral roots arising directly from the bearing root are collectively termed first-order roots, lateral roots arising from the first-order laterals are termed second-order roots; and so forth (Pagès et al., 1993; Pregitzer et al., 1997; Lecompte et al., 2001). In the second scheme (Fig. 1B), root order is assigned according to root hierarchal position. Here, lateral roots that bear no dependent laterals are collectively termed first-order roots, lateral roots that bear a single set of dependent first-order laterals are termed second-order roots; and so forth (Emmett et al., 2014; Polverigiani et al., 2014; Tawa and Takeda, 2015). In the latter approach, first-order roots are the most distally located roots in the branched hierarchy, while higher-order roots that support the lower-order laterals occur basally. The root systems of fruit crops may contain numerous branching orders; the higher the number, the more hierarchal levels of lateral roots depend upon the highest order. Rewald et al. (2011) noted as many as eight root orders in citrus, Valenzuela-Estrada et al. (2008) as many as seven in highbush blueberry, while a maximum of four have been noted for peach (Pagès et al., 1993) and between four and six for apple (Emmett et al., 2014; Polverigiani et al., 2014). In this review, we will mostly refer to root order according to the second scheme (Fig. 1B), unless otherwise indicated.

4. Root trait variation and fine root functionality with order

4.1. Development and growth of fine root functional types

All lateral roots start out as first-order absorptive roots. Most, but not necessarily all (Wilcox, 1968a; Pagès and Serra, 1994; Hodge et al., 2009), arise in acropetal succession at given distances from one another in longitudinal rows facing the internal protoxylem or phloem poles of the parent root (Lyford, 1980; Charlton, 1991; Thaler and Pagès, 1998; Vercambre et al., 2003; Jansen et al., 2013; Pagès, 2014). The internal vascular pattern of roots may conform to either one of four arrangements, depending on their order (Hishi and Takeda, 2005a, b; Tawa and Takeda, 2015). Roots with two protoxylem poles (diarch) tend to occupy the most distal positions within the branched hierarchy, whereas roots with four of more protoxylem poles (tetrarch or poly-arch) tend to occupy the most proximal positions within the branched

hierarchy. If the internal vascular pattern of a root conforms to a diarch arrangement (Fig. 2a), two lateral roots may arise between the xylem and phloem poles in the stele. If it conforms to a triarch (Fig. 2b) or tetrarch arrangement (Fig. 2c), three or four lateral roots may arise opposite the xylem poles in the stele and if it conforms to a poly-arch arrangement (Fig. 2d), five or more lateral roots may arise opposite the phloem poles in the stele (Sutton, 1980).

Not all lateral roots differentiate identically. In addition to differences in their anatomy and degree of mycorrhizal colonization, great variability in their morphology and growth behaviour have been reported. The variability in these traits overall have long been ascribed to the development of two functionally distinct root types in many tree species, namely "pioneer", "long" or "extension" roots and "fibrous", "short" or "absorptive" roots. This differentiation of the two mentioned root types can be observed in oil palm (Jourdan and Rey, 1997), rubber (Thaler and Pagès, 1998), cedar (Wilcox, 1962), oak (Lyford, 1980; Pagès, 1995), pine (Wilcox, 1968a, b; McCrady and Comerford, 1998), maple, walnut, aspen (Zadworny and Eissenstat, 2011) and black cottonwood (Bagniewska-Zadworna et al., 2012) and fruit crops like apple (Cripps, 1970; Atkinson, 1983; Emmett et al., 2014; Polverigiani et al., 2014), peach (Nightingale, 1935; Pagès et al., 1993), plum (Vercambre et al., 2003), citrus (Storey and Walker, 1987; Eissenstat and Achor, 1999) and olive (Polverigiani et al., 2011).

At the East Malling Research Station in Kent (UK), Atkinson (1983) assessed the ratio of short (fibrous) to long (pioneer) roots in the root system of apple trees during seasonal peaks in root production. In spring, although the total numbers of roots were minor compared to summer, most were pioneer roots. The ratio of fibrous to pioneer roots was highest during the summer peak, and compared to spring, higher in autumn when root numbers were decreasing. In apple orchards in Western Australia (Cripps, 1970), the ratio fibrous to pioneer roots was highest during the autumn peak. In spring, fibrous root numbers were lower compared to summer but pioneer root numbers did not differ markedly from summer. Although seasonal variations in the ratio of fibrous to pioneer roots in local apple orchards have been largely unexplored, it may bear some similarity to those in Western Australia since these two regions exhibit a similar seasonal pattern of fine root production. Yet, without further examination, this remains uncertain. As these two root types differ fundamentally in structure and function (sections 4.2 and 4.3), future studies on root nutrient (i.e. Ca) uptake dynamics in apple may benefit by including seasonal variations in the ratio of fibrous to pioneer roots as part of their investigations.

4.2. Variation in morphology and growth dynamics among individual roots within the fine root branching hierarchy

As the eventual progenitors of lower-order absorptive roots, pioneer roots represent a relatively small subset of first-order roots that are physiologically predetermined to become higher-order, semi-permanent members of the perennial root system (Atkinson, 1983; Persson, 2002; Waisel and Eshel, 2002; Wells and Eissenstat, 2003; Zadworny and Eissenstat, 2011). Based on the morphological measurements of these two root types in olive (Polverigiani et al., 2011), Polverigiani et al. (2014) considered all lateral fine roots upwards of the third order in 'M9' apple root systems as representative of the pioneer root fraction of the root system, and all first- and second order roots as representative of the fibrous root fraction. Since the diameter and specific root length (SRL; root length/dry mass) measurements of these two root types in olive fall within range of those reported previously for apple (Atkinson, 1983), this approximation seems valid.

In apple, fibrous roots are typically short and fine and of a relatively small diameter range of 0.3 – 1.1 mm (Atkinson, 1983; Polverigiani et al., 2014). By comparison, pioneer roots are long, straight and of a relatively large diameter range of 0.9 – 2.7 mm (Rogers, 1968; Atkinson, 1983). Furthermore, pioneer roots typically exhibit longer apical unbranched zones (Eissenstat and Achor, 1999), which can reach lengths of up to 50 – 100 mm depending on their growth rate (Wilcox, 1968a, b; Lyford, 1980; Sutton, 1980; Pagès et al., 1993). They also display a markedly lower SRL compared to fibrous roots (Polverigiani et al., 2011; Zadworny and Eissenstat, 2011). Other distinguishing features include the shape and size of the root tips. Although the tips of both root types appear white and translucent at emergence, the tips of pioneer roots usually appear pointed and swollen, having paraboloid-shaped meristematic zones, while those of fibrous roots appear small and rounded, having hemispherical or lens-shaped meristematic zones (Wilcox, 1968b; Lyford, 1980; Eissenstat and Achor, 1999; Majdi et al., 2001).

Root morphological- and growth rate measurements are strongly advised if a better understanding of root development and root system architecture is sought (Dupuy et al., 2010; Downie et al., 2015; Wang et al., 2015). Studies on root growth behaviour in monocots, such as maize (Pellerin and Tabourel, 1995; Pagès et al., 2010) and banana (Lecompte et al., 2001; Lecompte and Pagès, 2007), as well as dicots, such as cedar (Wilcox, 1962), pine (Wilcox,

1968a; Sutton, 1980), oak (Pagès and Serra, 1994; Pagès, 1995) and rubber (Thaler and Pagès, 1996, 1998), have shown that: (1) the initial apical diameter (representing the size of the apical meristem, measured in the conical part of the root apex at approximately 0.5 – 1 mm behind the root tip) of a new root at emergence is positively correlated with its growth potential and (2), the length of the apical unbranched zone (LAUZ; length visible from the root apex to the most distal lateral root) of a newly emerged root is linearly related to its elongation rate. While the initial apical diameter of a root reflects its potential growth rate, the actual growth rate as a function of the LAUZ is closer related to an increase in sink strength for available assimilates from the shoot (Pagès, 1995; Thaler and Pagès, 1996, 1998; Lecompte et al., 2001; Pagès et al., 2010). Thus, in light of the morphological differences between pioneer and fibrous roots, it should not be surprising that pioneer roots typically exhibit faster growth rates and longer growth duration at emergence compared with fibrous roots (Wilcox, 1962, 1968a; Lyford, 1980; Sutton, 1980; Persson, 2002; Waisel and Eshel, 2002; Zadworny and Eissenstat, 2011).

Lateral fine roots also vary in growth potential depending on their points of emergence relative to the base of the parent root, be it a primary taproot or a long lateral root (Wilcox, 1968a; Lyford, 1980; Pagès et al., 1993). In young peach seedlings for example, Pagès et al. (1993) noted a marked decline in growth rate among first-order roots with an increase in distance from the base of the taproot. The gradient in growth potential was particularly steep for pioneer roots in the basal 40 mm zone of the taproot, whereas most fibrous roots grew very little irrespective of their position along the taproot. Like the mean daily elongation rates of the roots of oak (Lyford, 1980; Pagès, 1995) and black cottonwood trees (Bagniewska-Zadworna et al., 2012), in peach (Pagès et al., 1993), pioneer roots may elongate at a rate of 4 – 8 mm day^{-1} in contrast to fibrous roots which have a maximum elongation rate of 2 – 3 mm day^{-1}. Moreover, fast-growing pioneer roots that emerge near the base of the taproot have a high initial growth rate that may be maintained for up to six weeks, while those that emerge closer to the apex have a lower initial growth rate that may be maintained for up to two weeks. By contrast, fast-growing fibrous roots stop elongating within two weeks of emergence, while the majority fibrous roots stop elongating within two days (Pagès et al., 1993).

Within the branched hierarchy of the tree root system, progressively higher (scheme 1: Wilcox, 1968a; Lyford, 1980; Pagès et al., 1993) or lower (scheme 2: Pregitzer et al., 2002; Vercambre et al., 2003; Guo et al., 2004) orders of branching show progressively lower growth rates and shorter growth duration. In the root systems of three-year-old plum trees, Vercambre et al.

(2003) noted that the main horizontal and vertical roots (previously pioneer roots) often reach lengths exceeding 1000 cm, while fifth-order branch roots may reach lengths of 10 to 100 cm, forth-order roots, 5 to 50 cm, third-order roots, 1 to 10 cm, second-order roots, 0.5 to 5 cm, and first-order roots, < 1 cm. Besides a progressive decline in root growth rate and duration with a decline in root order, these observations clearly indicate great variability in growth behaviour amongst roots within a given branching order. This variation may be ascribed to a combination of internal and external tree factors including: position of lateral root emergence; bearing root vigour; tree phenological stage; assimilate availability and growth conditions (Wilcox, 1968a; Pagès et al., 1993; Thaler and Pagès, 1996, 1998; Lecompte et al., 2001; Pagès et al., 2010). Thus, while root growth rate and duration may be a good indicator for distinguishing between pioneer and fibrous roots of the same age and order (i.e. newly emerged), on first observance, a more viable strategy may be to separate roots according to a combination of easily identifiable traits, including appearance (e.g. root tip morphology), diameter, SRL and average root length or LAUZ (Guo et al., 2004; Zadworny and Eissenstat, 2011; Polverigiani et al., 2014; Liu et al., 2018). Given the relationship between LAUZ and root growth rate, it may be helpful to know that this relationship is neither affected by the growth substrate nor growth conditions, and holds up whether comparing different branching orders or individual roots within one branching order (Pagès et al., 1993; Pellerin and Tabourel, 1995; Lecompte et al., 2001; Pagès et al., 2010).

4.3. Variation in anatomy, mycorrhizal colonization and physiological function among individual roots within the fine root branching hierarchy

4.3.1. Variation in anatomy and physiological function among root orders

Mounting evidence has shown that the hierarchal transition of lateral roots to a higher order (particularly from the second or third order upwards) is coupled with marked change in physiological function (Pregitzer et al., 2002; Wells and Eissenstat, 2003; Hishi, 2007; Guo et al., 2008b; Valenzuela-Estrada et al., 2008; Xia et al., 2010; Rewald et al., 2011; Long et al., 2013; McCormack et al., 2015). In general, root transport capacity increases, while root absorption capacity declines. This shift in function is mainly associated with ontogeny and secondary development within the root, involving the maturation and collapse of the cortex, secondary wall thickening, and the formation of secondary xylem and a continuous outer cork layer (CCL) or cork periderm. Together, these structural changes cause an increase in radial

resistance for the movement of water and nutrients across the root, and an increase in vascular conductance along the length of the root (Frensch et al., 1996; Steudle and Peterson, 1998), thereby, allowing the transport of a sufficient supply of absorbed water and nutrients (i.e. Ca) from the apical primary region of higher-order roots, as well as the primary developed, lower-order lateral roots to the shoot (Ferguson and Clarkson, 1975, 1976; Enstone et al., 2001; Cholewa and Peterson, 2004). Within the branched hierarchy of the apple root system, the most distal first-order roots consist entirely of primary developed roots, second-order roots consist of both primary and secondary developed roots, third-order roots consist mainly of secondary developed roots, and fourth- and higher-order roots consist entirely of secondary developed roots (Emmett et al., 2014). These trends, although similar to those found in various perennial tree species (Guo et al., 2008b; Huang et al., 2010; Long et al., 2013; Tawa and Takeda, 2015), including fruit crops like citrus (Eissenstat and Achor, 1999), are not universal. For example, in Manchurian ash (Xia et al., 2010), plum (Vercambre et al., 2003) and highbush blueberry (Valenzuela-Estrada et al., 2008), all roots of the first three orders are restricted to primary development and used primarily in resource absorption.

4.3.2. Variation in anatomy and physiological function among fine root functional types

In addition to characterizing fine roots by branching order, researchers commonly distinguish between fibrous and pioneer roots even if they are of the same order. At the expense of absorptive capacity and mycorrhizal colonization, larger diameter, pioneer roots are built to live longer, be less susceptible to abiotic and biotic stress factors, undergo high fibrous root branching and serve functions of soil exploration, resource transport and anchorage. By contrast, fibrous roots, particularly those of the first and second order, are relatively short-lived, often mycorrhizal and most retain their absorptive capacity for life (Atkinson, 1983; Wells and Eissenstat, 2003; Polverigiani et al., 2011; Zadworny and Eissenstat, 2011; Emmett et al., 2014). To find possible reasons for their early heterogeneity in function, Bagniewska-Zadworna et al. (2012) conducted a comparative temporal study on xylem vessel development in newly emerged first-order fibrous and pioneer roots of mature black cottonwood trees.

Consistent with their differential elongation rates, it was found that the formation of the primary xylem elements (protoxylem) occurred earlier and much closer to the tips of fibrous than pioneer roots in black cottonwood trees. Xylogenesis, however, appeared to proceed at a much faster rate in pioneer roots, as both root types had functional xylem vessels on the third to fourth

day of growth. Although the results of this study suggest that both root types can conduct water and nutrients to the shoot from an early age, they may not remain equally conductive. At six to seven days after emergence, the overall root hydraulic conductance of the primary xylem of pioneer roots was over a 100-fold greater than that of fibrous roots. Pioneer roots had developed twice the number of protoxylem poles, and twice the size, and four-fold the number of tracheary elements per xylem pole on average compared to fibrous roots. Moreover, in contrast to first-order pioneer roots where the first cambial divisions (i.e. secondary xylem development) were already noticed after seven to nine days of growth, first-order fibrous roots did not show any symptoms of secondary growth after nine, or even 21 days of growth. In agreement with Bagniewska-Zadworna et al. (2012), the timing and stages of vascular development in these two root types appear to be relatively consistent across species (Eissenstat and Achor, 1999; Zadworny and Eissenstat, 2011; Emmett et al., 2014). As previously suggested by Zadworny and Eissenstat (2011), Bagniewska-Zadworna et al. (2012) deduced that by the time pioneer roots start to branch, their greater investment in primary and secondary xylem allows unhindered transport of the increased influx of water and nutrients from the highly absorptive, lateral fibrous roots to the shoot.

In addition, pioneer and higher-order fibrous roots that have undergone secondary development and are, thus, better able to retain resources over winter (Zadworny et al., 2015), also act as important stores for carbohydrates (Persson, 2002; Guo et al., 2004; Polverigiani et al., 2011) and mineral nutrients such as N, P, potassium (K) and Ca (Conradie, 1990; Schreiner, 2005; Goebel et al., 2011; McCormack et al., 2012; Zadworny et al., 2015). These roots thus play a key role, especially in deciduous fruit crops such as apples, that rely on stored reserves to support root/shoot activity and growth at the beginning of the growing season (Tromp, 1983; Oliveira and Priestley, 1988; Cheng and Fuchigami, 2002). Results of local studies on potted (Terblanche, 1972; Terblanche et al., 1979; Van Zyl, 2016) and field-grown (Kangueehi, 2008; Wilsdorf, 2011) apple trees have also stressed the importance of permanent structures (including the roots, wood and bark) as major storage organs for Ca to be utilized by new growth (leaves, shoots and fruit) in spring. However, while it is well known that the root system of apple trees is important in the storage and remobilization of Ca, the contribution of the fine roots in relation to the whole root system has not yet been examined. Should Ca fertilizers, such as calcium nitrate ($Ca(NO_3)_2$), be applied to the soil in synchronization with seasonal peaks in fine root production (Wilsdorf, 2011; Van Zyl, 2016), the percentage of total Ca content in the different tissues of apple trees at the end of winter may be affected. In particular,

treatments such as defoliation or canopy scorching that could adversely affect new root production through reductions in below-ground C availability (Head, 1969; Chapin et al., 1990; Choi et al., 2003; Guo et al., 2004; Kwack et al., 2014), have been largely unexplored.

4.3.3. Variation in anatomy and mycorrhizal colonization among fine root functional types

The functional variability between fibrous and pioneer roots has also been addressed by including additional measures of anatomy and mycorrhizal colonization into studies. Since mycorrhizae are restricted to primary developed roots that have a viable and intact cortex (Schreiner, 2005; Resendes et al., 2008; Geldner, 2013; Taiz et al., 2015), a decrease in colonization with an increase in root order (Guo et al., 2008b; Valenzuela-Estrada et al., 2008; Xia et al., 2010; Long et al., 2013) is not surprising, as the senescence and loss of the cortex combined with the replacement of these tissues by a CCL invariably diminishes the ability of mycorrhizal fungi to colonize the root. More unexpected perhaps, is that newly emerged, first-order fibrous and pioneer roots may differ in their susceptibility to infection. Within the root systems of many hardwood tree species, such as oak (Wilcox, 1968b; Lyford, 1980), poplar (Hooker et al., 1992), spruce (Majdi et al., 2001), maple, walnut, aspen (Zadworny and Eissenstat, 2011) and Scots pine (Zadworny et al., 2015), arbuscular mycorrhizal (AM) colonization is strictly limited to fibrous roots. At less than 14 days after pioneer root emergence, Zadworny and Eissenstat (2011) found that these roots had a well-defined, multi-layered hypodermis in contrast to fibrous roots, whose hypodermis, depending on the species, consisted of only one or two cell layers. In addition, compared with fibrous roots, the exodermis of pioneer roots had a considerably lower percentage of passage cells, i.e. cells with no evidence of suberin deposition and secondary wall thickening (Peterson and Enstone, 1996; Ma and Peterson, 2003; Geldner, 2013). Similar observations have also been made on roots of various citrus species (Eissenstat and Achor, 1999). Since AM fungi penetrate the root exodermis of such species exclusively through passage cells (Peterson and Enstone, 1996; Sharda and Koide, 2008), variation in the extent of colonization among these two root types may, at least partly, be explained by differences in their anatomy, as was suggested by Zadworny and Eissenstat (2011).

The development of AM associations within the fine root architecture of apple trees may not be entirely limited to fibrous roots. Although AM fungi typically colonize fibrous roots in apple (Atkinson, 1983; Miller, 1983), pioneer roots have also been implicated (Miller, 1983). Besides

vesicles produced in roots in late-summer, various other AM fungal structures, including intraradical hyphae, hyphal coils and arbuscules, have been identified in fast-growing lateral roots within apple root systems in less than three or four days after their emergence in spring, summer and early-autumn (Resendes et al., 2008). In their study, mycorrhizae did not only selectively colonize fast-growing roots, which was rather unexpected, but also increased the rate and duration of growth of the colonized roots to the benefit of both the fungi and the host, presumably by enhanced defence production and altered root C demand (Hooker et al., 1992; Eissenstat et al., 2000; Hodge, 2009; Chen and Brassard, 2013). Whether the fast-growing root cohort in the study of Resendes et al. (2008) comprised both pioneer and fibrous roots is not known, but the above findings suggest that both root types may be susceptible to AM infection. In support, apple roots possess a uniseriate (single layer) exodermis that is non-lignified and only weakly suberized (Perumulla et al., 1990; Eissenstat et al., 2000).

In times of high nutrient demand, colonization of roots by AM fungi could improve plant performance by an increase in phosphorus (P) and N absorption (Bucher, 2007; Leigh et al., 2009; Fellbaum et al., 2012; Brzostek et al., 2014; Cheng et al., 2016; Van Geel et al., 2016). Evidence for such a role with Ca is scarce and limited to species other than apple. Although results are varied across species (Juice et al., 2006), in several hardwood tree species, including black cherry, green ash, red maple and sweetgum, AM inoculations under field conditions enhanced the Ca status of the roots, stems and leaves (Schultz and Kormanik, 1982). Although there is no direct evidence to support increased Ca capture by roots colonized with AM fungi, the results of Hooker et al. (1992) on poplar may provide some useful insights. In agreement with Resendes et al. (2008) in apple, Hooker et al. (1992) demonstrated that colonization of fine roots by AM fungi can directly alter root system morphology. Weekly additions of a balanced nutrient solution to the soil did not alter the morphology of any root order to as great an extent as AM colonization, the latter having caused large increases in individual root length and branching (i.e. in the number of new laterals produced per unit root length). Although further investigation is needed, AM fungi could possibly promote increased soil Ca uptake via changes in root system morphology, since the highest proportion of absorbed Ca is transported into the stele and subsequently the transpiration stream via the young, unsuberized regions of the root (Harrison-Murray and Clarkson, 1973; Robards et al., 1973; Ferguson and Clarkson, 1975, 1976; Cholewa and Peterson, 2004).

5. Root trait variation and fine root functionality with age

5.1. Root maturation and internal root developmental events

As a new lateral root emerges from a meristem, continued growth at the tip establishes an age gradient along its length by forming three anatomically distinct zones (Fig. 3). In order of increasing distance from the tip, the youngest, most apical zone is often described as the "white zone", followed immediately adjacent to it by the "condensed tannin" (CT) zone and later, the "cork" zone (Mckenzie and Peterson, 1995a, b; Taylor and Peterson, 2000; Enstone et al., 2001). Although root maturation (or ageing) is a natural occurrence, it is well understood that root growth rates (whether inherently controlled or under the control of the environment) determine the distances from the root tip at which maturation of the three zones take place (Peterson et al., 1999; Ma and Peterson, 2003; Enstone et al., 2003; Lux et al., 2004; Kumar et al., 2007; Song et al., 2011). In more slowly growing roots, tissue differentiation and/or maturation occurs closer to the tip (shortened white zone), and in faster growing roots, further from the tip (extended white zone). As these distances may vary considerably from root to root depending on the root type, climate (or season) and growth conditions (e.g. field-grown, pot-grown, pouch-grown or chamber-grown), the distances from the root tip given here for the various internal root developmental events merely serve as a guideline for future investigations.

5.1.1. White zone

In apple, as in many angiosperms and gymnosperms, the white zone of roots comprise several layers: an inner layer known as the endodermis; a mid-layer of succulent and functional central cortical parenchyma; and an external layer namely the epidermis and its subtending hypodermis (or exodermis) (Mackenzie, 1979; Lux et al., 2004; Geldner, 2013). The term "exodermis" refers to a hypodermis that has Casparian bands (Perumulla et al., 1990; Peterson and Perumulla, 1990; Meyer and Peterson, 2013). Depending on the root type and degree of mycorrhizal colonization (Sutton, 1980; Lyford, 1980), the epidermal layer of apple roots may bear many short root hairs (Head, 1966; Rogers, 1968; Miller, 1983). If present and functional, root hairs can markedly increase the uptake efficiency of nutrients like Ca (Peterson and Farquhar, 1996; Gilroy and Jones, 2000; Kumar et al., 2015). In the roots of many plant species (Damus et al., 1997; Ma and Peterson, 2003; Storey et al., 2003b; Cholewa and Peterson, 2004), including apple (Mackenzie, 1979; Perumulla et al., 1990), both hypodermal and endodermal cells develop Casparian bands. This stage of development is commonly referred to as "state I".

Exodermal Casparian bands generally develop much further from the root tip than their counterparts in the endodermis (Enstone and Peterson, 1992; Ma and Peterson, 2003; Cholewa and Peterson, 2004). In apple (Perumulla et al., 1990), they usually only develop after 60 – 100 mm from the root tip, whereas endodermal Casparian bands typically develop within 4 – 5 mm from the root tip (Mackenzie, 1979). Additionally, in apple (Riedhart and Guard, 1957; Mackenzie, 1979; Peterson et al., 1981; Weerdenburg and Peterson, 1983), as in many other plant species (Soukup et al., 2004; Pan et al., 2006; Fernandez-Garcia et al., 2009; Idris and Collings, 2015; Tawa and Takeda, 2015), roots develop heavy thickenings called "phi" thickenings, on the anticlinal walls of the cortical cells immediately toward the outside of the endodermis. In apple, these thickenings either develop simultaneously, or slightly in advance of both the deposition of Casparian bands within the primary walls of the endodermal cells, and the development and subsequent lignification of the first protoxylem elements in the stele (Riedhart and Guard, 1957; Mackenzie, 1979; Peterson et al., 1981; Weerdenburg and Peterson, 1983). To identify these structures via microscopy and histochemical staining (Schreiber et al., 1999; Enstone et al., 2001; Kumar et al., 2007; Idris and Collings, 2015), phi thickenings typically contain large quantities of cellulose and lignin and no suberin (Peterson et al., 1981; Weerdenburg and Peterson, 1983; Pratikakis et al., 1998; Fernandez-Garcia et al., 2009; Idris and Collings, 2015), whereas endodermal Casparian bands contain primarily lignin, in addition to a low but detectable amount of suberin (Schreiber et al., 1999; Ma and Peterson, 2003; Geldner, 2013; Lee et al., 2013; Meyer and Peterson, 2013).

Farther along the root axis, at about 16 mm basipetal to the root tip in apple (Mackenzie, 1979), the endodermal cells undergo "state II" development. Here, suberin lamellae are systematically laid down within the primary cell walls immediately adjacent to the plasma membranes of the endodermal cells facing the protophloem poles, while those facing the protoxylem poles remain in state I (Riedhart and Guard, 1957; Mackenzie, 1979), the latter cells termed endodermal "passage cells" (Peterson and Enstone, 1996; Martinka et al., 2012; Geldner, 2013). The suberin content of the affected cells are usually at least one order of magnitude higher than that of the unaffected cells (Schreiber et al., 1999). In this zone, the metaxylem, whose elements differentiate centripetally from the first protoxylem elements in the stele, is fully mature and it is also here where lateral roots are being initiated (Riedhart and Guard, 1957).

After approximately 30 mm from the root tip (Mackenzie, 1979), the endodermal cells undergo "state III" development. Here, lignified, non-suberized, carbohydrate or cellulosic cell walls

are deposited onto the state II suberin lamellae opposite the phloem poles only, while the passage cells opposite the xylem poles remain in place (Mackenzie, 1979; Peterson and Enstone, 1996; Schreiber et al., 1999; Ma and Peterson, 2003; Geldner, 2013). At the point of commencement of the CT zone (section 5.1.2), approximately 20 % of the root endodermal surface area may be accounted for by passage cells, as has been described for the roots of pine trees (Peterson et al., 1999). In pioneer and higher-order fibrous roots in particular (Emmett et al., 2014), the first vascular cambial divisions, which give rise to secondary xylem (wood) toward the inside and secondary phloem (bark) toward the outside of the stele (Peterson et al., 1999; Enstone et al., 2001; Chaffey, 2013; Lucas et al., 2013), become evident (Mackenzie, 1979). In addition, phi thickenings reach their maximum dimensions (Mackenzie, 1979; Weerdenburg and Peterson, 1983). The exodermis (if present) progressively matures basipetally (Enstone and Peterson, 1992; Peterson and Enstone, 1996; Cholewa and Peterson, 2004; Ma and Peterson, 2003; Geldner, 2013). In contrast to the Casparian bands in the endodermis; in apple, exodermal Casparian bands contain primarily suberin and no lignin (Perumulla et al., 1990) which, in addition to the subsequent deposition of suberin lamellae, may provide the root some protection against pathogen invasion and drying during times of stress (Kamula et al., 1994; Zimmerman and Steudle, 1998; Taleisnik et al., 1999; Hose et al., 2001; Enstone et al., 2003). It is thus important to note that the degree of exodermal development may change in response to the immediate environment (Zimmermann et al., 2000; Hishi et al., 2006; Meyer et al., 2009). The exodermis may conform to either one of two types. In species with a uniform (monomorphic) exodermis, such as maize, sunflower (Enstone and Peterson, 1992; Enstone et al., 2003) and grape (Storey et al., 2003b), all cells are elongated, but state I, II and III development are gradual and patchy. To the contrary, in species with a dimorphic exodermis, such as citrus (Walker et al., 1984; Storey and Walker, 1987; Eissenstat and Achor, 1999) and onion (Kamula et al., 1994; Ma and Peterson, 2000; Cholewa and Peterson, 2004), all cells undergo state I development, whereas only the long or elongated cells undergo state II development; the alternating short cells (state I) termed exodermal “passage cells” (Peterson and Enstone, 1996; Ma and Peterson, 2003; Geldner, 2013), remaining non-suberized. As few detailed studies on root exodermal development exist for apple, it is not clear whether apple roots tend to develop a uniform (monomorphic) or dimorphic exodermis. Because this has unique implications for the pathway of Ca^{2+} movement to the xylem (section 5.2.5), further investigation is needed.

5.1.2. Condensed tannin zone

Root browning may commence after approximately 50 mm from the root tip in apple (Nightingale, 1935). As this transition is gradual, the first clear division between the white and CT zones may only become evident after 70 – 110 mm from the root tip (Rogers, 1968; Mackenzie, 1979). The colour change associated with this transition is commonly ascribed to the centripetal and basipetal collapse of the epidermal and root cortical cells, the release and subsequent oxidation of phenolic compounds normally enclosed within the vacuole of the cells, and the deposition of brown-coloured, condensed tannins (proanthocyanidins) in their walls (Richards and Considine, 1981; Mckenzie and Peterson, 1995a; Peterson et al., 1999; Enstone et al., 2001). Concomitant with or slightly in advance of the collapse of these cells in pioneer and higher-order fibrous roots (Emmett et al., 2014), the pericycle (a layer of meristematic cells immediately toward the inside of the endodermis) enlarges and gives rise to the cork cambium from which cork cells are produced (Nightingale, 1935; Riedhart and Guard, 1957; Mckenzie and Peterson, 1995b). Periderm initiation, thus, becomes evident (Emmett et al., 2014). Secondary xylem development is also well advanced at this level (Nightingale, 1935; Riedhart and Guard, 1957; Mackenzie, 1979; Emmett et al., 2014) and may result in a 25 % higher stele: root diameter ratio (Weerdenburg and Peterson, 1983). Prior to the final collapse of the endodermis (which precedes the shedding of the cortex), the endodermal passage cells opposite the xylem poles rapidly undergo state II and III development (Mackenzie, 1979), thereby, substantially reducing the endodermal plasmalemma surface area of the CT zone (Enstone et al., 2001).

5.1.3. Cork zone

In apple, the cork zone may commence after approximately 130 – 170 mm from the root tip (Rogers, 1968; Mackenzie, 1979). As the root increases in girth, phi-thickenings become increasingly exposed as the cortical cells break apart and the endodermis gets crushed (Weerdenburg and Peterson, 1983). The cork cambium proceeds to produce a continuous layer of cork cells (Nightingale, 1935; Riedhart and Guard, 1957; Mackenzie, 1979) rich in suberin, lignin and tannin (Mckenzie and Peterson, 1995b; Peterson et al., 1999; Enstone et al., 2001), which ultimately forms the outermost protective layer of the root when the cortex (including the exodermis, cortical parenchyma, phi layer and endodermis) is shed. This renders the cork zone macroscopically similar to the CT zone, i.e. both zones appear brown in colour (Mckenzie and Peterson, 1995a, b; McCrady and Comerford, 1998; Peterson et al., 1999).

5.2. Calcium uptake and translocation in relation to internal root developmental stages

5.2.1. Apoplastic and symplastic flow

Root Ca uptake and translocation to the shoot has been extensively studied (Clarkson, 1984; McLaughlin and Wimmer, 1999; White, 2001; White and Broadley, 2003; Saure, 2005; Yang and Jie, 2005; Karley and White, 2009; Gilliham et al., 2011; Kumar et al., 2015). Calcium is absorbed from the soil solution and delivered to the shoot via the xylem either in its ionic form (Ca^{2+}) or in the form of chelates, such as Ca-malate, with the rate of deposition driven by transpiration and/or a growth-related demand, the cation exchange capacity in the xylem (and apoplast) and the solution (i.e. soil Ca^{2+}) concentration (Biddulph et al., 1961; Bell and Biddulph, 1963; Tromp, 1975; Ferguson and Bollard, 1976; Van de Geijn and Petit, 1979; Atkinson et al., 1992; Del Amor and Marcelis, 2003, 2006). Upon contact with the root surface, Ca^{2+} ions move radially across the root to the xylem either rapidly by mass flow and diffusion through the extracellular spaces or cell walls (apoplastic pathway), or less rapidly through the cytoplasm of individually linked cells via plasmodesmata (symplastic pathway). Calcium ions can also follow a coupled transcellular or cell-to-cell pathway, involving both apoplastic and symplastic flow. As the latter pathway is very costly in terms of energy for transport, it is the least preferred (Clarkson, 1993). Although the relative contributions of the pathways for Ca^{2+} delivery to the xylem are still largely unknown, evidence suggests that during periods of high shoot demand, e.g. in apple, around six weeks after bud break during the shoot extension phase (late-spring to early-summer) and after harvest until leaf drop in winter (Terblanche, 1972; Hanekom, 1973; Kangueehi, 2008), the apoplastic pathway is preferentially utilized in regions of the root that are unsuberized (Harrison-Murray and Clarkson, 1973 in marrow; Ferguson and Clarkson, 1976 in barley). As the cytosolic free Ca^{2+} concentration of all root cells must be maintained at a low (sub micromolar) level in order for roots to perceive and coordinate appropriate physiological responses to an array of environmental, developmental or pathological stimuli (Gilroy et al., 1993; Sanders et al., 1999, 2002; Amtmann and Blatt, 2009; McAinsh and Pittman, 2009; Lautner and Fromm, 2010; Hocking et al., 2016), it has been suggested that the extracellular pathway would enable roots to meet the high demands of the shoot for Ca without compromising intracellular Ca^{2+} signalling (White, 1998, 2001; White and Broadley, 2003). In contrast, during periods of low shoot demand (or under conditions of reduced transpiration), the highest proportion of Ca^{2+} may be transported via the symplast (Engels, 1999 in maize; Baxter et al., 2009 in *Arabidopsis*); the latter, more selective pathway

allowing the root to control the rate of Ca^{2+} delivery into the xylem based on the demand for Ca in the shoot (Clarkson, 1993; White, 1998, 2001; White and Broadley, 2003; Wang et al., 2006a). To date, this has yet to be demonstrated for apple.

5.2.2. White zone without Casparian bands

Studies on the roots of barley (Russell and Sanderson, 1967), maize (Ferguson and Clarkson, 1975) and onion (Cholewa and Peterson, 2004) showed that Ca^{2+} ions move through the apoplast as far as the central stellar parenchyma cells in the root tip. As this is the only region of the root without complete endodermal Casparian bands (Mackenzie, 1979), these findings may likely apply to apple. This zone of the root also lacks functional xylem vessels in the stele (Mackenzie, 1979). Consequently, little Ca^{2+} translocation from the root tip to the shoot is anticipated, based on previous findings (Russell and Sanderson, 1967; Ferguson and Clarkson, 1975; Cholewa and Peterson, 2004). To regulate Ca levels in this region of the root, a large fraction of absorbed Ca^{2+} may accumulate as raphide crystals in the vacuoles of specialised root cortical cells, known as crystal idioblasts (Storey et al., 2003a; Franceschi and Nakata, 2005). Between 3 – 5 mm (Peterson et al., 1981; Weerdenburg and Peterson, 1983) and 60 – 100 mm (Perumulla et al., 1990) from the root tip, Ca^{2+} ions may move freely through diffusion, radially into the root through the epidermis and central cortex via the apoplast (Peterson and Enstone, 1996; Enstone et al., 2003). Prior to reaching the endodermis, apoplastic Ca^{2+} ions must first cross the lignified phi layer. Even though phi thickenings develop in close proximity to the endodermal Casparian bands (Mackenzie, 1979), they do not function as a major apoplastic barrier to ions (Peterson et al., 1981; Weerdenburg and Peterson, 1983; Perumulla et al., 1990). Their specific role in plant roots has yet to be established, but phi thickenings are generally believed to function in root structural support and protection against environmental stresses (Idris and Collings, 2015).

5.2.3. White zone with endodermal Casparian bands

Between 4 – 5 mm and 16 mm from the root tip (Mackenzie, 1979), the uptake of Ca^{2+} ions from the cortical apoplast into the endodermis are progressively, although not necessarily completely, blocked by Casparian bands nestled between the endodermal cells (Clarkson, 1993; White, 2001; White and Broadley, 2003; Ranathunge et al., 2005). Consequently, the majority apoplastic Ca^{2+} ions are redirected into the cytoplasm of the endodermal cells through

plasma membrane-embedded Ca^{2+}-permeable channels located on the cortical side of the Casparian band (White et al., 2000; Miedema et al., 2001; Wang et al., 2006a; McAinsh and Pittman, 2009; Yang et al., 2011). To deliver Ca^{2+} to the xylem at the necessary rate whilst maintaining steady-state Ca^{2+} levels in the cytoplasm of the endodermal cells, Ca^{2+} ions are actively pumped from the symplast into the stellar apoplast by energy-dependent, plasma membrane-embedded Ca^{2+} transporters (Ca^{2+}-ATPases) located on the stellar side of the Casparian band (Cholewa and Peterson, 2004; Hayter and Peterson, 2004; Hong-Qiang et al., 2004; Wang et al., 2006a; McAinsh and Pittman, 2009; Yang et al., 2011). Since state II development are yet to commence (Mackenzie, 1979), all endodermal cells are available for Ca^{2+} influx into the symplast and Ca^{2+} efflux from the symplast into the stellar apoplast. Moreover, based on the findings of others (Russell and Sanderson, 1967; Robards et al., 1973; Ferguson and Clarkson, 1975; Cholewa and Peterson, 2004), a substantial fraction of absorbed Ca^{2+} may be translocated to the shoot from this zone, as the stele now contains functional xylem vessels (Riedhart and Guard, 1957; Mackenzie, 1979).

5.2.4. White zone with endodermal suberin lamellae and cellulosic secondary cell walls

In the roots of many plant species, including marrow (Harrison-Murray and Clarkson, 1973), barley (Robards et al., 1973; Ferguson and Clarkson, 1976), maize (Ferguson and Clarkson, 1975) and onion (Cholewa and Peterson, 2004), Ca^{2+} translocation to the shoot is progressively reduced by the deposition of suberin lamellae, and in some species, cellulosic secondary walls in the endodermis. Even though these developments, which respectively occur after 16 and 30 mm from the root tip in apple (Mackenzie, 1979), do not interrupt the plasmodesmata that link the endodermis to the central cortex and stele, at least in some species (Harrison-Murray and Clarkson, 1973; Robards et al., 1973; Ma and Peterson, 2000, 2001; White, 2001), they severely restrict direct access of nutrient ions like Ca^{2+} to the plasma membranes of the endodermal cells (Harrison-Murray and Clarkson, 1973; Robards et al., 1973; Walker et al., 1984). Thus, assuming Ca^{2+} movement in the symplast is slow or does not take place to any significant extent (Ferguson and Clarkson, 1976; White, 1998, 2001; White and Broadley, 2003), the main sites of entry for apoplastic Ca^{2+} ions into the stellar apoplast are the unsuberized endodermal passage cells that face the protoxylem poles in the stele (Walker et al., 1984; Peterson and Enstone, 1996; Hayter and Peterson, 2004). At these sites, further penetration towards the stele proceeds as described above. In this zone of the root, other possible sites of entry are breaks in the endodermis caused by the emergence of lateral root

primordia originating in the pericycle (Ferguson and Clarkson, 1975; Sanderson, 1983; Zimmerman and Steudle, 1998; White, 2001). This apoplastic bypass for Ca^{2+} movement into the stele is, however, temporary. Firstly, the deposition of suberin lamellae in the endodermal cells immediately surrounding the developing lateral root proceeds more rapidly than in the neighbouring endodermal cells further away (Martinka et al., 2012), and secondly, Casparian bands deposited at the base of the developing lateral root rapidly interconnects with those of the endodermis of the parent root (Weerdenburg and Peterson, 1983; Peterson and Enstone, 1996; Martinka et al., 2012). Thus, in comparison with the first pathway (via state I passage cells), the latter temporary pathway for Ca^{2+} influx into the xylem would most likely not contribute significantly to Ca^{2+} translocation to the shoot.

5.2.5. White zone with exodermal Casparian bands and suberin lamellae

As has been described by others (Peterson and Enstone, 1996; Ma and Peterson, 2003; Meyer and Peterson, 2013), in apple (Perumulla et al., 1990), hypodermal cells may develop Casparian bands and suberin lamellae in close succession after 60 – 100 mm from the root tip. Like that in the endodermis, these developments present an important apoplastic barrier to the movement of nutrient ions into the walls of the central cortical cells (Robards et al., 1973; Ferguson and Clarkson, 1975; Zimmermann et al., 2000; Hose et al., 2001; Cholewa and Peterson, 2004; Baxter et al., 2009). Since aliphatic suberin is the main reason for the hydrophobic properties of apoplastic barriers (Schreiber et al., 1999, 2005; Franke and Schreiber, 2007; Ranathunge and Schreiber, 2011), the presence of such a barrier may be especially important for apple roots whose exodermal Casparian bands contain primarily suberin (Perumulla et al., 1990; Eissenstat et al., 2000). To confirm this, a comparative analysis of the magnitude of Ca^{2+} influx into the root, and the quantity, chemical nature and microstructure of the suberin deposits in the exodermis is advised (Schreiber et al., 2005; Franke and Schreiber, 2007; Ranathunge and Schreiber, 2011). Because it is uncertain whether apple roots possess a uniform (monomorphic) or dimorphic exodermis, for this review, the possible influence of both on Ca^{2+} influx into the central cortex will be discussed.

In roots with a uniform exodermis, Ca^{2+} ions enter the epidermis and central cortex via the apoplast in areas where the exodermis is still absent or immature (Peterson and Enstone, 1996; Enstone et al., 2003). In its mature state, the exodermis very severely restricts apoplastic Ca^{2+} movement to the central cortex (Ferguson and Clarkson, 1975), but not symplastic movement,

as state I, II and III development do not interrupt the plasmodesmata that link the exodermis to the epidermis and central cortex (Clarkson et al., 1987). In roots with a dimorphic exodermis, the major sites for Ca^{2+} entry into the central cortex are the unsuberized exodermal passage cells or "short cells" (Walker et al., 1984; Peterson and Enstone, 1996; Ma and Peterson, 2000, 2001; Hayter and Peterson, 2004). As the rapid deposition of suberin lamellae in the alternating "long cells" completely sever plasmodesmata that initially linked them to their adjacent epidermal (if still present) and central cortical cells (Walker et al., 1984; Ma and Peterson, 2000, 2001), Ca^{2+} ions already in the symplast may enter the central cortex through intact plasmodesmata in the "short cells" (Ma and Peterson, 2000). For apoplastic Ca^{2+} ions, the mechanism for entry into the walls of the central cortical cells is like that described for the endodermis (Ma and Peterson, 2001; Cholewa and Peterson, 2004; Hayter and Peterson, 2004). In this zone of the root, newly emerged lateral roots may also provide temporary points of ingress to the cortex through breaks in the exodermis (Enstone and Peterson, 1992; Zimmerman and Steudle, 1998); these breaks having been shown, are more permeable to solutes than those at the endodermis (Zimmerman and Steudle, 1998).

5.2.6. Condensed tannin and cork zones

The transition of roots from white to brown presents its own consequences for Ca absorption. Although root browning is associated with a marked decline in respiration and root active metabolism (Comas et al., 2000; Bouma et al., 2001; Volder et al., 2005; Baldi et al., 2010; Rewald et al., 2014), it may not be indicative of a total loss of functionality (Wang et al., 1995; Wells and Eissenstat, 2001; Anderson et al., 2003; Yao et al., 2006). However, compared to young white roots or root tips, the CT zone invariably exhibits a much lower absorption capacity, which is a result of a reduction in the cortical plasmalemma surface area (CPSA; the combined plasmalemma surface areas of the various cortical cell layers with direct access to the soil solution) of the root. Indeed, upon collapse of the epidermis and central cortical cells, nutrient ions destined for the xylem are only able to access the stellar apoplast and enter the transpiration stream via state I endodermal passage cells; these cells being the only ones with a plasma membrane directly exposed to the external soil solution (Mckenzie and Peterson, 1995a, b; Peterson and Enstone, 1996; Peterson et al., 1999; Taylor and Peterson, 2000; Enstone et al., 2001; Kumar et al., 2007). As state II and III development of the endodermal passage cells, together with the shedding of the cortex, progressively reduce the CPSA available for ion uptake to nil, it has been suggested that the cork zone of roots is incapable of

nutrient absorption (Peterson et al., 1999; Taylor and Peterson, 2000; Enstone et al., 2001; Kumar et al., 2007). However, based on the results of studies on nutrient uptake by older, "woody" roots in citrus, grape (Crider, 1933), apple, cherry (Atkinson and Wilson, 1979, 1980) and slash pine (Escamilla and Comerford, 2000), the permeability of the cork zone likely varies with species and/or the nutrient element being absorbed. With respect to Ca absorption in apple, Atkinson and Wilson (1980) found no significant differences in uptake between the white and "woody" roots of the trees. It is, however, uncertain whether the observed Ca uptake patterns were justified on an anatomical scale, that is whether the "woody" roots in their study were in the CT stage or the cork stage. Therefore, to further our understanding of the processes and patterns of Ca uptake along the length of apple roots, physiological measurements of ion uptake should be correlated with the anatomy of the different root zones, as has been suggested by Mckenzie and Peterson (1995a, b) and Peterson et al. (1999).

To achieve this, researchers must be able to distinguish between the different root zones. While it is relatively easy to visually distinguish white from brown roots, the border between the CT zone and the cork zone cannot be detected with the naked eye (Mckenzie and Peterson, 1995b; Escamilla and Comerford, 2000; Enstone et al., 2001; Kumar et al., 2007). Unless roots are white (primary developed), or brown and > 2 or 2.5 mm in diameter (secondary developed), neither root colour nor root diameter are reliable predictors of root developmental stages (McCrady and Comerford, 1998; Rewald et al., 2014). This is especially true for roots that develop in non-homogeneous media (Enstone et al., 2001). Future investigations should, thus, rather be based on an extended root classification system. This would involve separating roots according to order, colour and anatomy (Hishi, 2007; Rewald et al., 2014; McCormack et al., 2015; Freschet and Roumet, 2017). The anatomical parameters used to distinguish primary developed from secondary developed brown (or woody) roots also requires careful consideration (McCrady and Comerford, 1998; Enstone et al., 2001; Kumar et al., 2007). In the root systems of various woody perennials, Comerford et al. (1994) and Escamilla and Comerford (2000) classified roots as "woody" if both the vascular cambium and secondary xylem elements external to the metaxylem in the stele could be observed. However, it is not clear whether a CCL was present. As the latter almost definitely constitutes a major apoplastic barrier for ion uptake (Mckenzie and Peterson, 1995a, b), recognizing its presence may be a better anatomical approach to substantiate the possible differential dynamics of Ca^{2+} movement across the brown, primary developed roots with passage cells and the woody, secondary developed roots without passage cells and with a CCL, in apple.

6. Fine root lifespan and nutrient uptake efficiency with age and order

During most of the growing season, apple roots either remain white for several days before elimination (either by rapid decomposition or by herbivory) or turn partially brown after about two to three weeks before naturally decaying and disappearing or continue to turn light brown and then permanently brown after about four to six weeks. These roots may either persist in the soil for several months to well over a year before natural decomposition commences, or may continue to increase in diameter to become woody permanent members of the root system (Nightingale, 1935; Head, 1966; Rogers, 1968; Atkinson and Wilson, 1980; Atkinson, 1983; Psarras et al., 2000; Ma et al., 2013). At the Agricultural Research Centre in Rock Springs, Pennsylvania, Wells and Eissenstat (2001) showed that of all the autumn-born fine roots of apple trees that survived winter, 3 – 12 % were < 0.3 mm in diameter (the majority of which were white), 30 % were between 0.3 – 0.5 mm in diameter (less than half of which were brown) and 55 – 60 % were > 0.5 mm in diameter (the majority of which were brown and viable and gave rise to new white lateral roots in spring). The median lifespans of the roots were between 30 – 60 days for those < 0.3 mm in diameter, between 100 and 150 days for those between 0.3 – 0.5 mm in diameter, and > 200 days for those > 0.5 mm in diameter.

Many intrinsic and extrinsic plant factors are known to influence fine root lifespan. These include factors such as the species and rootstock genotype (Black et al., 1998; Eissenstat et al., 2000; Yao et al., 2006), rooting depth and number of neighbouring roots (Wells and Eissenstat, 2001; Wells et al., 2002a; Anderson et al., 2003; Baddeley and Watson, 2005), mycorrhizal symbiosis (Hooker et al., 1995; Guo et al., 2008b; Resendes et al., 2008), soil-born pathogen- and herbivory pressure (Kosola et al., 1995; Wells et al., 2002b; Emmett et al., 2014), tree age and season of root formation (Wang et al., 2006b; Guo et al., 2008b; Gu et al., 2011; Hou et al., 2012), plant growth rate and C status (Psarras et al., 2000; Comas and Eissenstat, 2004; McCormack et al., 2012), soil water and nutrient (i.e. N) availability (Pregitzer et al., 1993, 1995; Majdi et al., 2001; Adams et al., 2013), canopy, yield and groundcover management (Head, 1966, 1969; Atkinson, 1972; Eissenstat and Duncan, 1992; Yao et al., 2009) along with elevated temperature and atmospheric carbon dioxide (CO_2) levels (Pregitzer et al., 1995; Stover et al., 2010; McCormack et al., 2013). Yet, the results of Wells and Eissenstat (2001) and those of others in apple (Yao et al., 2009; Hou et al., 2012), clearly showed that fine root lifespan is strongly affected by fine root diameter. A strong positive correlation between fine root lifespan and diameter has also been reported in grape (Anderson et al., 2003), peach (Wells

et al., 2002a), wild cherry (Baddeley and Watson, 2005) and various other temperate tree species and shrubs (Huang et al., 2010; Gu et al., 2011; McCormack et al., 2012). Contingent on the range of root diameter variation across branching orders, the position of fine roots within the branched hierarchy of the root system may be an equally important (Wells et al., 2002a; Gu et al., 2011; McCormack et al., 2012) or possibly more important determinant of fine root lifespan than fine root diameter (Withington et al., 2006; Guo et al., 2008b; Valenzuela-Estrada et al., 2008; Xia et al., 2010). Considering the species-specific relationship between fine root lifespan and diameter, it is widely accepted that the most distal, non-woody branch orders have the shortest lifespans (or highest turnover rates). In apple, this applies to all roots of the first order and a large fraction of roots of the second order (Emmett et al., 2014).

Like the relationship between fine root lifespan and diameter in some species, fine root lifespan and branching order in most, is closely linked with changes in fine root traits, including morphology, physiology, anatomy and chemistry (Weemstra et al., 2016). Although these correlations may vary across species and environments, an increase in fine root lifespan with an increase in fine root diameter and/or branching order has most consistently been associated with a decrease in SRL, an increase in root tissue density (RTD; root dry mass: volume ratio), an increase in the number of xylem vessels per root cross-sectional area, an increase in xylem and/or stele: root diameter ratio, an increase in total non-structural carbohydrate (TNC; starch and soluble sugar) and cellulose concentrations, a decrease in tissue N concentration, an increase in TNC: tissue N concentration ratio and an increase in tissue Ca content (Pregitzer et al., 1997, 2002; Guo et al., 2004; Wang et al., 2006b; Valenzuela-Estrada et al., 2008; Huang et al., 2010; Li et al., 2010; Xia et al., 2010; McCormack et al., 2012; Jia et al., 2013). Compared to the relatively long-lived (or perennial), larger diameter and/or higher-order roots that represent the resource transport and conservation fraction of the fine root population, the relatively short-lived (or ephemeral), smaller diameter and/or lower-order terminal roots that represent the resource acquisition fraction of the fine root population generally exhibit significantly higher resource uptake and respiration rates (Pregitzer et al., 1998; Desrochers et al., 2002; Volder et al., 2005; Sun and Mao, 2011; Jia et al., 2013; Rewald et al., 2014; Miyatani et al., 2018). These rates rapidly decline when roots turn brown with age (Comas et al., 2000; Bouma et al., 2001; Baldi et al., 2010). In terms of C expended in root construction and maintenance, larger diameter and/or higher-order roots are more expensive to construct and less expensive to maintain based on their higher C:N ratios and lower SRL and tissue N concentrations. These high C:N ratios are associated with the accumulation of phenolic/defence

compounds, such as lignin, tannin and suberin, along with storage compounds, such as starch and soluble sugars, the development of a thick exodermis (at least in some species) and the formation of secondary xylem, in addition to a CCL. Alternatively, smaller diameter and/or lower-order terminal roots are less expensive to construct and more expensive to maintain based on their lower C:N ratios and higher SRL and tissue N concentrations, the latter associated with the repair and replacement of proteins and enzymes that facilitate ion uptake and assimilation (Eissenstat, 1992; Eissenstat and Yanai, 1997; Pregitzer et al., 1997, 1998, 2002; Eissenstat and Volder, 2005; Volder et al., 2005).

By regulating the lifespan of different portions of their root system, continuous shifts in the total amount of fine roots present as well as the age structure of the fine root population may offer plants some control over their efficiency of soil resource acquisition. Indeed, larger diameter and/or higher-order roots that bear many daughter roots are more likely to be retained for extended periods to justify their construction costs and support the lateral roots that depend on them. In contrast, smaller diameter and/or lower-order, terminal roots that bear few or no daughter roots are more likely only maintained until their lifetime efficiency in terms of resources gained (benefit) over C expended (cost) is maximized (Eissenstat et al., 2000; Eissenstat and Yanai, 1997, 2002; Wells and Eissenstat, 2003; Hodge, 2004; Eissenstat and Volder, 2005; Chen and Brassard, 2013). Studies on citrus (Bouma et al., 2001), apple (Bouma et al., 2001; Eissenstat et al., 2001) and grape (Volder et al., 2005), all of which exhibit contrasting root characteristics and fine root lifespans, confirmed that the optimal lifespan at which the lifetime efficiency of nutrient acquisition is maximized and a root should be shed, is more dependent on changes in root nutrient uptake. These changes are then closely associated with nutrient depletion zones developing around the root and a decline in root physiological capacity with age, rather than with changes in respiratory C costs, as the latter in any event, is unlikely to remain high in an older root exhibiting reduced uptake rates (Bouma et al., 2001; Eissenstat et al., 2001; Volder et al., 2005). These results, thus, suggest that in times of high nutrient demand, roots with diminished uptake rates would most probably not be maintained, but instead be replaced with new roots that are highly active and cheaper to construct, in more favourable soil locations, such as localized nutrient-rich patches. The ability and speed of deployment of new roots in such zones, however, is likely to be influenced by C availability (Lavely et al., 2018). Furthermore, provided that water and nutrients (i.e. N) remain in ample supply, studies on apple (Eissenstat et al., 2001) and various other hardwood tree species (Pregitzer et al., 1993) have shown that plants can extend the longevity (and therefore uptake

potential) of their most efficient (lower-order) roots. This is achieved, presumably through increased carbohydrate allocation which, depending on the C status of the (higher-order) roots that support them, could be supplied by export of stored carbohydrate reserves (Guo et al., 2004).

To summarize: in contrast to apple growing regions in the northern hemisphere where trees experience severe winters and early leaf drop in autumn, apple trees established in the warmer growing regions of the Western Cape tend to exhibit an extended period of active white root growth during autumn and into winter, which could be ascribed to conducive soil conditions (i.e. soil temperature and moisture levels) as well as the presence of an abundant amount of actively photosynthesizing leaves until late into autumn (Van Zyl, 2016). It was, thus, concluded that fine root production and growth in locally grown apple trees during autumn and into winter could be sustained by both current photosynthates and stored carbohydrate reserves (Van Zyl, 2016), which agree with the findings of others (Pregitzer et al., 1993; Eissenstat et al., 2001; Guo et al., 2004; Lavely et al., 2018) provided soil nutrients (i.e. N) remain in ample supply. Since new white roots are best equipped for high Ca uptake, future studies on Ca uptake and distribution in apple could benefit by including tree canopy manipulation and variation in soil nutrient, i.e. $Ca(NO_3)_2$ supply in their investigations.

7. Conclusion

Within the fine root architecture of trees, a distribution of roots that differ in development (pioneer vs. fibrous roots), age (primary developed white and brown roots vs. secondary developed brown or woody roots) and lifespan (short-lived or ephemeral roots vs. long-lived or perennial roots) exist, both within and between branching orders. Potentially, each of these fine root categories portray functional significance with regards to soil Ca uptake and transport in apple. It is thus of common opinion that their associated traits, which include measures of morphology, anatomy, chemistry, physiology and mycorrhizal associations, be captured when investigating soil resource acquisition (Freschet and Roumet, 2017; McCormack et al., 2017).

To improve our understanding of root Ca uptake in apple as is required for local growing conditions, standardized sampling methods are needed in order to capture the links between fine root function (i.e. absorption vs. transport and storage) and root trait variation among these fine root categories. Based on general evidence, we propose intact fine root branches be

separated according to the steps laid out in Table 1. This implicates setting up a tentative framework for future investigations on root Ca uptake in apple, which could be extended or adapted as our current knowledge of the linkages among fine root categories, traits and functions in apple expand.

References

Adams, T.S., Mccormack, M.L., Eissenstat, D.M., 2013. Foraging strategies in trees of different root morphology: The role of root lifespan. Tree Physiol. 33, 940–948.

Amtmann, A., Blatt, M.R., 2009. Regulation of macronutrient transport. New Phytol. 181, 35–52.

An, H., Dong, H., Wu, T., Wang, Y., Xu, X., Zhang, X., 2017. Root growth angle: An important trait that influences the deep rooting of apple rootstocks. Sci. Hort. 216, 256–263.

Anderson, L.J., Comas, L.H., Lakso, A.N., Eissenstat, D.M., 2003. Multiple risk factors in root survivorship: A 4-year study in Concord grape. New Phytol. 158, 489–501.

Atkinson, C.J., Ruiz, L.P., Mansfield, T.A., 1992. Calcium in xylem sap and the regulation of its delivery to the shoot. J. Exp. Bot. 43, 1315–1324.

Atkinson, D., 1972. Seasonal periodicity of black current root growth and the influence of simulated mechanical harvesting. J. Hort. Sci. 47, 165–172.

Atkinson, D., 1974. Some observations on the distribution of root activity in apple trees. Plant Soil 40, 333–342.

Atkinson, D., 1983. The growth, activity and distribution of the fruit tree root system. Plant Soil 71, 23–35.

Atkinson, D., Wilson, S.A., 1979. The root-soil interface and its significance for fruit tree roots of different ages, in: Harley, J.L., Russel, R.S. (Eds.), The Soil-Root Interface. Academic Press, London, pp. 259–271.

Atkinson, D., Wilson, S.A., 1980. The growth and distribution of fruit tree roots: Some consequences for nutrient uptake, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 137–150.

Baddeley, J.A., Watson, C.A., 2005. Influences of root diameter, tree age, soil depth and season on fine root survivorship in *Prunus avium*. Plant Soil 276, 15–22.

Bagniewska-Zadworna, A., Byczyk, J., Eissenstat, D.M., Oleksyn, J., Zadworny, M., 2012. Avoiding transport bottlenecks in an expanding root system: Xylem vessel development in fibrous and pioneer roots under field conditions. Am. J. Bot. 99, 1417–1426.

Baldi, E., Wells, C.E., Marangoni, B., 2010. Nitrogen absorption and respiration in white and

brown peach roots. J. Plant Nutr. 33, 461–469.

Bardgett, R.D., Mommer, L., De Vries, F.T., 2014. Going underground: Root traits as drivers of ecosystem processes. Trends Ecol. Evol. 29, 692–699.

Baxter, I., Hosmani, P.S., Rus, A., Lahner, B., Borevitz, J.O., Muthukumar, B., Mickelbart, M. V., Schreiber, L., Franke, R.B., Salt, D.E., 2009. Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in *Arabidopsis*. PLoS Genet. 5, 1–12.

Bell, C.W., Biddulph, O., 1963. Translocation of calcium. Exchange versus mass flow. Plant Physiol. 38, 610–614.

Bianco, R.L., Policarpo, M., Scariano, L., 2003. Effects of rootstock vigour and in-row spacing on stem and root growth, conformation and dry-matter distribution of young apple trees. J. Hort. Sci. Biotechnol. 78, 828–836.

Biddulph, O., Nakayama, F.S., Cory, R., 1961. Transpiration stream and ascension of calcium. Plant Physiol. 36, 429–436.

Black, K.E., Harbron, C.G., Franklin, M., Atkinson, D., Hooker, J.E., 1998. Differences in root longevity of some tree species. Tree Physiol. 18, 259–264.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Brzostek, E.R., Fisher, J.B., Phillips, R.P., 2014. Modelling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. J. Geophys. Res. Biogeosciences 119, 1684–1697.

Bucher, M., 2007. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol. 173, 11–26.

Chaffey, N., 2013. Secondary growth of tree roots: Cytoskeletal perspectives, in: Eshel, A., Beeckman, T. (Eds.), Plant Roots: The Hidden Half. CRC Press, Boca Raton-London-New York, p. 8.1-8.19.

Chapin, F.S., Schulze, E., Mooney, H.A., 1990. The ecology and economics of storage in plants. Annu. Rev. Ecol. Syst. 21, 423–447.

Charlton, W.A., 1991. Lateral root initiation, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant

Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 103–148.

Chen, H.Y.H., Brassard, B.W., 2013. Intrinsic and extrinsic controls of fine root life span. Crit. Rev. Plant Sci. 32, 151–161.

Cheng, L., Chen, W., Adams, T.S., Wei, X., Li, L., McCormack, M.L., Deforest, J.L., Koide, R.T., Eissenstat, D.M., 2016. Mycorrhizal fungi and roots are complementary in foraging within nutrient patches. Ecology 97, 2815–2823.

Cheng, L., Fuchigami, L.H., 2002. Growth of young apple trees in relation to reserve nitrogen and carbohydrates. Tree Physiol. 22, 1297–1303.

Chiatante, D., Beltotto, M., Onelli, E., Iorio, A. Di, Montagnoli, A., Scippa, S.G., 2010. New branch roots produced by vascular cambium derivatives in woody parental roots of *Populus nigra* L. Plant Biosyst. 144, 420–433.

Chiatante, D., Scippa, G.S., Iorio, A. Di, De Micco, V., Sarnataro, M., 2007. Lateral root emission in woody taproots of *Fraxinus ornus* L. Plant Biosyst. 141, 204–213.

Choi, S.T., Park, D.S., Song, W.D., Kang, S.M., Shon, G.M., 2003. Effect of different degrees of defoliation on fruit growth and reserve accumulation in young 'Fuyu' trees. Acta Hort. 601, 99–104.

Cholewa, E., Peterson, C.A., 2004. Evidence for symplastic involvement in the radial movement of calcium in onion roots. Plant Physiol. 134, 1793–1802.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

Clarkson, D.T., Robards, A.W., Stephens, J.E., Stark, M., 1987. Suberin lamellae in the hypodermis of maize (*Zea mays*) roots; development and factors affecting the permeability of hypodermal layers. Plant Cell Environ. 10, 83–93.

Comas, L.H., Eissenstat, D.M., 2004. Linking fine root traits to maximum potential growth rate among 11 mature temperate tree species. Funct. Ecol. 18, 388–397.

Comas, L.H., Eissenstat, D.M., Lakso, A.N., 2000. Assessing root death and root system dynamics in a study of grape canopy pruning. New Phytol. 147, 171–178.

Comerford, N.B., Smethurst, P.J., Escamilla, J.A., 1994. Nutrient uptake by woody root

systems. New Zeal. J. For. Sci. 24, 195–212.

Conradie, W.J., 1990. Distribution and translocation of nitrogen absorbed during late spring by two-year-old grapevines grown in sand culture. Am. J. Enol. Vitic. 41, 241–250.

Crider, F.J., 1933. Selective absorption of ions not confined to young rootlets. Science 78, 169.

Cripps, J.E.L., 1970. A seasonal pattern of apple root growth in western Australia. J. Hort. Sci. 45, 153–161.

Damus, M., Peterson, R.L., Enstone, D.E., Peterson, C.A., 1997. Modifications of cortical cell walls in roots of seedless vascular plants. Bot. Acta 110, 190–195.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hort. Rev. 40, 107–146.

De Silva, H.N., Hall, A.J., Tustin, D.S., Gandar, P.W., 1999. Analysis of distribution of root length density of apple trees on different dwarfing rootstocks. Ann. Bot. 83, 335–345.

Del Amor, F.M., Marcelis, L.F.M., 2003. Regulation of nutrient uptake, water uptake and growth under calcium starvation and recovery. J. Hort. Sci. Biotechnol. 78, 343–349.

Del Amor, F.M., Marcelis, L.F.M., 2006. Differential effect of transpiration and Ca supply on growth and Ca concentration of tomato plants. Sci. Hort. 111, 17–23.

Desrochers, A., Landhäusser, S.M., Lieffers, V.J., 2002. Coarse and fine root respiration in aspen (*Populus tremuloides*). Tree Physiol. 22, 725–732.

Downie, H.F., Adu, M.O., Schmidt, S., Otten, W., Dupuy, L.X., White, P.J., Valentine, T.A., 2015. Challenges and opportunities for quantifying roots and rhizosphere interactions through imaging and image analysis. Plant Cell Environ. 38, 1213–1232.

Dupuy, L., Gregory, P.J., Bengough, A.G., 2010. Root growth models: Towards a new generation of continuous approaches. J. Exp. Bot. 61, 2131–2143.

Eissenstat, D.M., 1992. Costs and benefits of constructing roots of small diameter. J. Plant Nutr. 15, 763–782.

Eissenstat, D.M., Achor, D.S., 1999. Anatomical characteristics of roots of citrus rootstocks that vary in specific root length. New Phytol. 141, 309–321.

Eissenstat, D.M., Bauerle, T.L., Comas, L.H., Neilsen, D., Neilsen, G.H., Lakso, A.N., Smart, D.R., 2006. Seasonal patterns of root growth in relation to shoot phenology in grape and

apple. Acta Hort. 721, 21–26.

Eissenstat, D.M., Duncan, L.W., 1992. Root growth and carbohydrate responses in bearing citrus trees following partial canopy removal. Tree Physiol. 10, 245–257.

Eissenstat, D.M., Volder, A., 2005. The efficiency of nutrient acquisition over the life of a root, in: Bassirirad, H. (Ed.), Nutrient Acquisition by Plants. Springer, Berlin Heidelberg, pp. 185–220.

Eissenstat, D.M., Wells, C.E., Wang, L., 2001. Root efficiency and mineral nutrition in apple. Acta Hort. 564, 165–183.

Eissenstat, D.M., Wells, C.E., Yanai, R.D., Whitbeck, J.L., 2000. Building roots in a changing environment: Implications for root longevity. New Phytol. 147, 33–42.

Eissenstat, D.M., Yanai, R.D., 1997. Ecology of root life span. Adv. Ecol. Res. 27, 1–60.

Eissenstat, D.M., Yanai, R.D., 2002. Root life span, efficiency, and turnover, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 221–238.

Emmett, B., Nelson, E.B., Kessler, A., Bauerle, T.L., 2014. Fine-root system development and susceptibility to pathogen colonization. Planta 239, 325–340.

Engels, C., 1999. Regulation of xylem transport of calcium from roots to shoot of maize by growth-related demand. J. Plant Nutr. Soil Sci. 162, 287–294.

Enstone, D.E., Peterson, C.A., 1992. The apoplastic permeability of root apices. Can. J. Bot. 70, 1502–1512.

Enstone, D.E., Peterson, C.A., Hallgren, S.W., 2001. Anatomy of seedling tap roots of loblolly pine (*Pinus taeda* L.). Trees 15, 98–111.

Enstone, D.E., Peterson, C.A., Ma, F., 2003. Root endodermis and exodermis: Structure, function, and responses to the environment. J. Plant Growth Regul. 21, 335–351.

Escamilla, J.A., Comerford, N.B., 2000. Phosphorus and potassium uptake by woody roots of twelve-year-old slash pine trees. For. Ecol. Manage. 129, 153–166.

Fan, W., Yang, H., 2011. Effect of soil type on root architecture and nutrient uptake by roots of young apple rootstocks. Acta Hort. 903, 885–890.

Fellbaum, C.R., Gachomo, E.W., Beesetty, Y., Choudhari, S., Strahan, G.D., Pfeffer, P.E.,

Kiers, E.T., Bucking, H., 2012. Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. Proc. Natl. Acad. Sci. 109, 2666–2671.

Ferguson, I.B., Bollard, E.G., 1976. The movement of calcium in woody stems. Ann. Bot. 40, 1057–1065.

Ferguson, I.B., Clarkson, D.T., 1975. Ion transport and endodermal suberization in the roots of *Zea mays*. New Phytol. 75, 69–79.

Ferguson, I.B., Clarkson, D.T., 1976. Simultaneous uptake and translocation of magnesium and calcium in barley (*Hordeum vulgare* L.) roots. Planta 128, 267–269.

Fernandez-Garcia, N., Lopez-Perez, L., Hernandez, M., Olmos, E., 2009. Role of phi cells and the endodermis under salt stress in *Brassica oleracea*. New Phytol. 181, 347–360.

Fitter, A., 2002. Characteristics and functions of root systems, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 15–32.

Fitter, A.H., Stickland, T.R., Harvey, M.L., Wilson, G.W., 1991. Architectural analysis of plant root systems. 1. Architecturals correlates of exploitation effiency. New Phytol. 118, 375–382.

Franceschi, V.R., Nakata, P.A., 2005. Calcium oxalate in plants: formation and function. Annu. Rev. Plant Biol. 56, 41–71.

Franke, R., Schreiber, L., 2007. Suberin - a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. 10, 252–259.

Frensch, J., Hsiao, T.C., Steudle, E., 1996. Water and solute transport along developing maize roots. Planta 198, 348–355.

Freschet, G.T., Roumet, C., 2017. Sampling roots to capture plant and soil functions. Funct. Ecol. 31, 1506–1518.

Geldner, N., 2013. The endodermis. Annu. Rev. Plant Biol. 64, 531–558.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Gilroy, S., Bethke, P.C., Jones, R.L., 1993. Calcium homeostasis in plants. J. Cell Sci. 106, 453–461.

Gilroy, S., Jones, D.L., 2000. Through form to function: Root hair development and nutrient uptake. Trends Plant Sci. 5, 56–60.

Goebel, M., Hobbie, S., Bulaj, B., Zadworny, M., Archibald, D., Oleksyn, J., Reich, P., Eissenstat, D.M., 2011. Decomposition of the finest root branching orders: linking carbon and nutrient dynamics belowground to fine root function and structure. Ecol. Monogr. 81, 89–102.

Green, S.R., Clothier, B.E., 1999. The root zone dynamics of water uptake by a mature apple tree. Plant Soil 206, 61–77.

Gu, J., Wei, X., Wang, J., Dong, X., Wang, Z., 2015. Marked differences in standing biomass, length density, anatomy and physiological activity between white and brown roots in *Fraxinus mandshurica* Rupr. plantation. Plant Soil 392, 267–277.

Gu, J., Yu, S., Sun, Y., Wang, Z., Guo, D., 2011. Influence of root structure on root survivorship: An analysis of 18 tree species using a minirhizotron method. Ecol. Res. 26, 755–762.

Guo, D., Xia, M., Wei, X., Chang, W., Liu, Y., Wang, Z., 2008a. Anatomical traits associated with absorption and mycorrhizal colonization are linked to root branch order in twenty-three Chinese temperate tree species. New Phytol. 180, 673–683.

Guo, D., Mitchell, R.J., Withington, J.M., Fan, P., Hendricks, J.J., 2008b. Endogenous and exogenous controls of root life span, mortality and nitrogen flux in a longleaf pine forest: root branch order predominates. J. Ecol. 96, 737–745.

Guo, D.L., Mitchell, R.J., Hendricks, J.J., 2004. Fine root branch orders respond differentially to carbon source-sink manipulations in a longleaf pine forest. Oecologia 140, 450–457.

Hanekom, A.N., 1973. Opname van kalsium-45 deur appelbome by verskillende vogpeile en die induksie van biterpit. Ph.D. dissertation, Faculty of Natural Science, Randse Afrikaanse Universiteit.

Harrison-Murray, R.S., Clarkson, D.T., 1973. Relationships between structural development and the absorption of ions by the root system of *Cucurbita pepo*. Planta 114, 1–16.

Hayter, M.L., Peterson, C.A., 2004. Can Ca^{2+} fluxes to the root xylem be sustained by Ca^{2+}-ATPases in exodermal and endodermal plasma membranes? Plant Physiol. 136, 4318–4325.

Head, G.C., 1966. Estimating seasonal changes in the quantity of white suberized root on fruit trees. J. Hort. Sci. 41, 197–206.

Head, G.C., 1967. Effects of seasonal changes in shoot growth on the amount of unsuberized root on apple and plum trees. J. Hort. Sci. 42, 169–180.

Head, G.C., 1969. The effects of fruiting and defoliation on seasonal trends in new root production on apple trees. J. Hort. Sci. 44, 175–181.

Hishi, T., 2007. Heterogeneity of individual roots within the fine root architecture: Causal links between physiological and ecosystem functions. J. For. Res. 12, 126–133.

Hishi, T., Takeda, H., 2005a. Life cycles of individual roots in fine root system of *Chamaecyparis obtusa* Sieb. et Zucc. J. For. Res. 10, 181–187.

Hishi, T., Takeda, H., 2005b. Dynamics of heterorhizic root systems: Protoxylem groups within the fine-root system of *Chamaecyparis obtusa*. New Phytol. 167, 509–521.

Hishi, T., Tateno, R., Takeda, H., 2006. Anatomical characteristics of individual roots within the fine-root architecture of *Chamaecyparis obtusa* (Sieb. & Zucc.) in organic and mineral soil layers. Ecol. Res. 21, 754–758.

Hocking, B., Tyerman, S.D., Burton, R.A., Gilliham, M., 2016. Fruit Calcium: Transport and Physiology. Front. Plant Sci. 7, 1–17.

Hodge, A., 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytol. 162, 9–24.

Hodge, A., 2009. Root decisions. Plant Cell Environ. 32, 628–640.

Hodge, A., Berta, G., Doussan, C., Merchan, F., Crespi, M., 2009. Plant root growth, architecture and function. Plant Soil 321, 153–187.

Hong-Qiang, Y., Yu-Ling, J., Lian-Zhong, Z., Ming-Gang, C., 2004. The effect of IBA on the Ca^{2+} absorption and Ca^{2+}-ATPase activity and their ultracytochemical localization in apple roots. Acta Hort. 636, 211–219.

Hooker, J.E., Black, K.E., Perry, R.L., Atkinson, D., 1995. Arbuscular mycorrhizal fungi induced alteration to root longevity of poplar. Plant Soil 172, 327–329.

Hooker, J.E., Munro, M., Atkinson, D., 1992. Vesicular-arbuscular mycorrhizal fungi induced alteration in poplar root system morphology. Plant Soil 145, 207–214.

Hose, E., Clarkson, D.T., Steudle, E., Schreiber, L., Hartung, W., 2001. The exodermis: A variable apoplastic barrier. J. Exp. Bot. 52, 2245–2264.

Hou, C., Ma, L., Luo, F., Wang, Y., Zhang, X., Han, Z., 2012. Impact of rootstock and interstems on fine root survivorship and seasonal variation in apple. Sci. Hort. 148, 169–176.

Hu, Y., Zhao, J., Shen, X., Mao, Z., Sun, F., Zhang, Y., Shu, H.R., 2011. Apple tree development affected by soil texture and vertical soil volume. Acta Hort. 903, 891–896.

Huang, G., Zhao, X., Zhao, H., Huang, Y., Zuo, X., 2010. Linking root morphology, longevity and function to root branch order: A case study in three shrubs. Plant Soil 336, 197–208.

Hughes, K.A., Gandar, P.W., 1993. Length densities, occupancies and weights of appe root systems. Plant Soil 148, 211–221.

Idris, N.A., Collings, D.A., 2015. The life of phi: the development of phi thickenings in roots of the orchids of the genus *Miltoniopsis*. Planta 241, 489–506.

Jansen, L., Demeulenaere, M., Beeckman, T., 2013. Lateral root development, in: Eshel, A., Beeckman, T. (Eds.), Plant Roots: The Hidden Half. CRC Press, Boca Raton-London-New York, p. 6.1-6.12.

Jia, S., Mclaughlin, N.B., Gu, J., Li, X., Wang, Z., 2013. Relationships between root respiration rate and root morphology, chemistry and anatomy in *Larix gmelinii* and *Fraxinus mandshurica*. Tree Physiol. 33, 579–589.

Jourdan, C., Rey, H., 1997. Architecture and development of the oil-palm (*Elaeis guineensis* Jacq.) root system. Plant Soil 189, 33–48.

Juice, S.M., Fahey, T.J., Siccama, T.G., Driscoll, C.T., Denny, E.G., Eagar, C., Cleavitt, N.L., Minocha, R., Richardson, A.D., 2006. Response of sugar maple to calcium addition to northern hardwood forest. Ecology 87, 1267–1280.

Kamula, S.A., Peterson, C.A., Mayfield, C.I., 1994. The plasmalemma surface area exposed to the soil solution is markedly reduced by maturation of the exodermis and death of the epidermis in onion roots. Plant Cell Environ. 17, 1183–1193.

Kangueehi, G.N., 2008. Nutrient requirement and distribution of intensively grown 'Brookfield Gala' apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Karley, A.J., White, P.J., 2009. Moving cationic minerals to edible tissues: potassium,

magnesium, calcium. Curr. Opin. Plant Biol. 12, 291–298.

Kosola, K.R., Eissenstat, D.M., Graham, J.H., 1995. Root demography of mature citrus trees: the influence of *Phytophthora nicotianae*. Plant Soil 171, 283–288.

Kumar, A., Singh, U.M., Manohar, M., Gaur, V.S., 2015. Calcium transport from source to sink: understanding the mechanism(s) of acquisition, translocation, and accumulation for crop biofortification. Acta Physiol. Plant. 37, 1722–1736.

Kumar, P., Hallgren, S.W., Enstone, D.E., Peterson, C.A., 2007. Root anatomy of *Pinus taeda* L.: Seasonal and environmental effects on development in seedlings. Trees 21, 693–706.

Kwack, Y., Kim, H.L., Chae, W., Kim, S.H., Lee, Y., Kim, J.G., 2014. Carbohydrate reserves of non-fruiting young kiwifruit vines as affected by early artificial defoliation. Hort. Environ. Biotechnol. 55, 462–470.

Lalibert, E., 2017. Below-ground frontiers in trait-based plant ecology. New Phytol. 213, 1597–1603.

Lautner, S., Fromm, J., 2010. Calcium-dependent physiological processes in trees. Plant Biol. 12, 268–274.

Lavely, E.K., Zhang, J., Adams, T.S., Bryla, D.R., Deforest, J.L., Marini, R.P., Crassweller, R., Eissenstat, D.M., 2018. Root and mycorrhizal fungal foraging responses to fruit removal in apple trees. Plant Soil. doi:10.1007/s11104-018-3773-8

Lecompte, F., Ozier-Lafontaine, H., Pagès, L., 2001. The relationships between static and dynamic variables in the description of root growth. Consequences for field interpretation of rooting variability. Plant Soil 236, 19–31.

Lecompte, F., Pagès, L., 2007. Apical diameter and branching density affect lateral root elongation rates in banana. Environ. Exp. Bot. 59, 243–251.

Lee, Y., Rubio, M.C., Alassimone, J., Geldner, N., 2013. A mechanism for localized lignin deposition in the endodermis. Cell 153, 402–412.

Leigh, J., Hodge, A., Fitter, A.H., 2009. Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. New Phytol. 181, 199–207.

Li, A., Guo, D., Wang, Z., Liu, H., 2010. Nitrogen and phosphorus allocation in leaves, twigs, and fine roots across 49 temperate, subtropical and tropical tree species: A hierarchical pattern. Funct. Ecol. 24, 224–232.

Liu, Y., Wang, G., Yu, K., Li, P., Xiao, L., Liu, G., 2018. A new method to optimize root order classification based on the diameter interval of fine root. Sci. Rep. 8, 1–11.

Long, Y., Kong, D., Chen, Z., Zeng, H., 2013. Variation of the linkage of root function with root branch order. PLoS One 8, 1–11.

Lucas, W.J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S.R., Helariutta, Y., He, X.Q., Fukuda, H., Kang, J., Brady, S.M., Patrick, J.W., Sperry, J., Yoshida, A., Lopez-Millan, A., Grusak, M.A., Kachroo, P., 2013. The Plant Vascular System: Evolution, Development and Functions. J. Integr. Plant Biol. 55, 294–388.

Lux, A., Luxova, M., Abe, J., Morita, S., 2004. Root cortex: Structural and functional variability and responses to environmental stress. Root Res. 13, 117–131.

Lyford, W.H., 1980. Development of the root system of northern red oak (*Quercus rubra* L.). Harvard Forest Paper No. 21, Petersham, MA, USA.

Ma, F., Peterson, C.A., 2000. Plasmodesmata in onion (*Allium cepa* L.) roots: A study enabled by improved fixation and embedding techniques. Protoplasma 211, 103–115.

Ma, F., Peterson, C.A., 2001. Frequencies of plasmodesmata in *Allium cepa* L. roots: Implications for solute transport pathways. J. Exp. Bot. 52, 1051–1061.

Ma, F., Peterson, C.A., 2003. Current insights into the development, structure, and chemistry of the endodermis and exodermis of roots. Can. J. Bot. 81, 405–421.

Ma, L., Hou, C.W., Zhang, X.Z., Li, H.L., Han, D., Wang, Y., Han, Z.H., 2013. Seasonal growth and spatial distribution of apple tree roots on different rootstocks or interstems. J. Amer. Soc. Hort. Sci. 138, 79–87.

Mackenzie, K.A.D., 1979. The development of the endodermis and phi layer of apple roots. Protoplasma 100, 21–32.

Majdi, H., Damm, E., Nylund, J., 2001. Longevity of mycorrhizal roots depends on branching order and nutrient availability. New Phytol. 150, 195–202.

Martinka, M., Dolan, L., Pernas, M., Abe, J., Lux, A., 2012. Endodermal cell - cell contact is required for the spatial control of Casparian band development in *Arabidopsis thaliana*. Ann. Bot. 110, 361–371.

McAinsh, M.R., Pittman, J.K., 2009. Shaping the calcium signature. New Phytol. 181, 275–294.

McCormack, M.L., Adams, T.S., Smithwick, E.A.H., Eissenstat, D.M., 2012. Predicting fine root lifespan from plant functional traits in temperate trees. New Phytol. 195, 823–831.

McCormack, M.L., Dickie, I.A., Eissenstat, D.M., Fahey, T.J., Fernandez, C.W., Guo, D., Helmisaari, H., Hobbie, E.A., Iversen, C.M., Jackson, R.B., Leppalammi-Kujansuu, J., Norby, R.J., Phillips, R.P., Pregitzer, K.S., Pritchard, S.G., Rewald, B., Zadworny, M., 2015. Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes. New Phytol. 207, 505–518.

McCormack, M.L., Eissenstat, D.M., Prasad, A.M., Smithwick, E.A.H., 2013. Regional scale patterns of fine root lifespan and turnover under current and future climate. Glob. Chang. Biol. 19, 1697–1708.

McCormack, M.L., Guo, D., Iversen, C.M., Chen, W., Eissenstat, D.M., Fernandez, C.W., Li, L., Ma, C., Ma, Z., Poorter, H., Reich, P.B., Zadworny, M., Zanne, A., 2017. Building a better foundation: Improving root-trait measurements to understand and model plant and ecosystem processes. New Phytol. 215, 27–37.

McCrady, R.L., Comerford, N.B., 1998. Morphological and anatomical relationships of loblolly pine fine roots. Trees 12, 431–437.

McCully, M.E., 1999. Roots in soil: Unearthing the Complexities of Roots and their rhizospheres. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 695–718.

Mckenzie, B.E., Peterson, C.A., 1995a. Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 1. Anatomy and permeability of the white and tannin zones. Bot. Acta 108, 127–137.

Mckenzie, B.E., Peterson, C.A., 1995b. Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 2. Anatomy and permeability of the cork zone. Bot. Acta 108, 138–143.

McLaughlin, S.B., Wimmer, R., 1999. Calcium physiology and terrestrial ecosystem processes. New Phytol. 142, 373–417.

Meyer, C.J., Peterson, C.A., 2013. Structure and function of three suberized cell layers: Epidermis, exodermis and endodermis, in: Eshel, A., Beeckman, T. (Eds.), Plant Roots: The Hidden Half. CRC Press, Boca Raton-London-New York, p. 5.1-5.20.

Meyer, C.J., Seago, J.L., Peterson, C.A., 2009. Environmental effects on the maturation of the endodermis and multiseriate exodermis of *Iris germanica* roots. Ann. Bot. 103, 687–702.

Miedema, H., Bothwell, J.H.F., Brownlee, C., Davies, J.M., 2001. Calcium uptake by plant cells - channels and pumps acting in concert. Trends Plant Sci. 6, 514–519.

Miller, D.D., 1983. Identification and physiological significance of mycorrhizal associations of apple (*Malus domestica* Borkh.). Retrospective Theses and Dissertations, Iowa State University. Paper 8941.

Miyatani, K., Tanikawa, T., Makita, N., Hirano, Y., 2018. Relationships between specific root length and respiration rate of fine roots across stands and seasons in *Chamaecyparis obtusa*. Plant Soil 423, 215–227.

Nightingale, G.T., 1935. Effects of temperature on growth, anatomy, and metabolism of apple and peach roots. Bot. Gaz. 96, 581–639.

Oliveira, C.M., Priestley, C.A., 1988. Carbohydrate reserves in deciduous fruit trees. Hort. Rev. 10, 403–430.

Pagès, L., 1995. Growth patterns of the lateral roots of young oak (*Quercus robur*) tree seedlings. Relationship with apical diameter. New Phytol. 130, 503–509.

Pagès, L., 2014. Branching patterns of root systems: Quantitative analysis of the diversity among dicotyledonous species. Ann. Bot. 114, 591–598.

Pagès, L., Kervella, J., Chadoeuf, J., 1993. Development of the root system of young peach trees (*Prunus persica* L. Batsch): A morphometrical analysis. Ann. Bot. 71, 369–375.

Pagès, L., Serra, V., 1994. Growth and branching of the taproot of young oak trees - a dynamic study. J. Exp. Bot. 45, 1327–1334.

Pagès, L., Serra, V., Draye, X., Doussan, C., Pierret, A., 2010. Estimating root elongation rates from morphological measurements of the root tip. Plant Soil 328, 35–44.

Pan, C.X., Nakao, Y., Nii, N., 2006. Anatomical development of phi thickening and the Casparian strip in loquat roots. J. Japanese Soc. Hort. Sci. 75, 445–449.

Paolillo, D.J., Bassuk, N.L., 2005. On the occurrence of adventitious branch roots on root axes of trees. Am. J. Bot. 92, 802–809.

Pellerin, S., Tabourel, F., 1995. Length of the apical unbranched zone of maize axile roots: Its relationship to root elongation rate. Environ. Exp. Bot. 35, 193–200.

Persson, H.A., 2002. Root systems of arboreal plants, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp.

187–204.

Perumulla, C.J., Peterson, C.A., Enstone, D.E., 1990. A survey of angiosperm species to detect hypodermal Casparian bands. I. Roots with a uniseriate hypodermis and epidermis. Bot. J. Linn. Soc. 103, 93–112.

Peterson, C.A., Emanuel, M.E., Weerdenburg, C.A., 1981. The permeability of phi thickenings in apple (*Pyrus malus*) and geranium (*Pelargonium hortorum*) roots to an apoplastic fluorescent dye tracer. Can. J. Bot. 59, 1107–1110.

Peterson, C.A., Enstone, D.E., 1996. Functions of passage cells in the endodermis and exodermis of roots. Physiol. Plant. 97, 592–598.

Peterson, C.A., Enstone, D.E., Taylor, J.H., 1999. Pine root structure and its potential significance for root function. Plant Soil 217, 205–213.

Peterson, C.A., Perumulla, C.J., 1990. A survey of angiosperm species to detect hypodermal Casparian bands. II. Roots with a multiseriate hypodermis and epidermis. Bot. J. Linn. Soc. 103, 113–125.

Peterson, R.L., Farquhar, M.L., 1996. Root hairs: Specialized tubular cells extending root surfaces. Bot. Rev. 62, 1–40.

Pierret, A., Moran, C.J., Doussan, C., 2005. Conventional detection methodology is limiting our ability to understand the roles and functions of fine roots. New Phytol. 166, 967–980.

Polverigiani, S., Kelderer, M., Neri, D., 2014. Growth of 'M9' apple root in five Central Europe replanted soils. Plant Root 8, 55–63.

Polverigiani, S., Massetani, F., Neri, D., Perilli, A., Kelderer, M., Rainer, A., 2013. Effect of four different soil management techniques on apple root development. Acta Hort. 1001, 361–367.

Polverigiani, S., McCormack, M.L., Mueller, C.W., Eissenstat, D.M., 2011. Growth and physiology of olive pioneer and fibrous roots exposed to soil moisture deficits. Tree Physiol. 31, 1228–1237.

Pratikakis, E., Rhizopoulou, S., Psaras, G.K., 1998. A phi layer in roots of *Ceratonia siliqua* L. Bot. Acta 111, 93–98.

Pregitzer, K.S., 2002. Fine roots of trees: A new perspective. New Phytol. 154, 267–270.

Pregitzer, K.S., Deforest, J.L., Burton, A.J., Allen, M.F., Ruess, W.R., Hendrick, R.L., 2002.

Fine root architecture of nine North American trees. Ecol. Monogr. 72, 293–309.

Pregitzer, K.S., Hendrick, R.L., Fogel, R., 1993. The demography of fine roots in response to patches of water and nitrogen. New Phytol. 125, 575–580.

Pregitzer, K.S., King, J.S., Burton, A.J., Brown, S.E., 2000. Responses of tree fine roots to temperature. New Phytol. 147, 105–115.

Pregitzer, K.S., Kubiske, M.E., Yu, C.K., Hendrick, R.L., 1997. Relationships among root branch order, carbon, and nitrogen in four temperate species. Oecologia 111, 302–308.

Pregitzer, K.S., Laskowski, M.J., Burton, A.J., Lessard, V.C., Zak, D.R., 1998. Variation in sugar maple root respiration with root diameter and soil depth. Tree Physiol. 18, 665–670.

Pregitzer, K.S., Zak, D.R., Curtis, P.S., Kubiske, M.E., Teeri, J.A., Vogel, C.S., 1995. Atmospheric CO_2, soil nitrogen and turnover of fine roots. New Phytol. 129, 579–585.

Psarras, G., Merwin, I.A., Lakso, A.N., Ray, J.A., 2000. Root growth phenology, root longevity, and rhizosphere respiration of field grown 'Mutsu' apple trees on 'Malling 9' rootstock. J. Am. Soc. Hort. Sci. 125, 596–602.

Ranathunge, K., Schreiber, L., 2011. Water and solute permeabilities of *Arabidopsis* roots in relation to the amount and composition of aliphatic suberin. J. Exp. Bot. 62, 1961–1974.

Ranathunge, K., Steudle, E., Lafitte, R., 2005. A new precipitation technique provides evidence for the permeability of Casparian bands to ions in young roots of corn (*Zea mays* L.) and rice (*Oryza sativa* L.). Plant Cell Environ. 28, 1450–1462.

Resendes, M.L., Bryla, D.R., Eissenstat, D.M., 2008. Early events in the life of apple roots: Variation in root growth rate is linked to mycorrhizal and nonmycorrhizal fungal colonization. Plant Soil 313, 175–186.

Rewald, B., Ephrath, J.E., Rachmilevitch, S., 2011. A root is a root is a root? Water uptake rates of citrus root orders. Plant Cell Environ. 34, 33–42.

Rewald, B., Rechenmacher, A., Godbold, D.L., 2014. It's complicated: Intraroot system variability of respiration and morphological traits in four deciduous tree species. Plant Physiol. 166, 736–745.

Richards, D., Considine, J.A., 1981. Suberization and browning of grapevine roots, in: Brouwer, R., Gasparikova, O., Kolek, J., Loughman, B.C. (Eds.), Structure and Function of Plant Roots. Dr. W. Junk, London, pp. 111–115.

Riedhart, J.M., Guard, A.T., 1957. On the anatomy of the roots of apple seedlings. Bot. Gaz. 118, 191–194.

Robards, A.W., Jackson, S.M., Clarkson, D.T., Sanderson, J., 1973. The structure of barley roots in relation to the transport of ions into the stele. Protoplasma 77, 291–311.

Rogers, W.S., 1968. Amount of cortical and epidermal tissue shed from roots of apple. J. Hort. Sci. 43, 527–528.

Russell, R.S., Sanderson, J., 1967. Nutrient uptake by different parts of the intact roots of plants. J. Exp. Bot. 18, 491–508.

Sanders, D., Brownlee, C., Harper, J.F., 1999. Communicating with calcium. Plant Cell 11, 691–706.

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F., 2002. Calcium at the crossroads of signaling. Plant Cell 14, S401–S417.

Sanderson, J., 1983. Water uptake by different regions of the barley root. Pathways of radial flow in relation to development of the endodermis. J. Exp. Bot. 34, 240–253.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: Its mechanism and endogenous control. Sci. Hort. 105, 65–89.

Schreiber, L., Franke, R., Hartmann, K.D., Ranathunge, K., Steudle, E., 2005. The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix). J. Exp. Bot. 56, 1427–1436.

Schreiber, L., Hartmann, K., Skrabs, M., Zeier, J., 1999. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. J. Exp. Bot. 50, 1267–1280.

Schreiner, R.P., 2005. Spatial and temporal variation of roots, arbuscular mycorrhizal fungi, and plant and soil nutrients in a mature Pinot Noir (*Vitis vinifera* L.) vineyard in Oregon, USA. Plant Soil 276, 219–234.

Schultz, R.C., Kormanik, P.P., 1982. Vesicular-arbuscular mycorrhiza and soil fertility influence mineral concentrations in seedlings of eight hardwood species. Can. J. For. Res. 12, 829–834.

Sharda, J.N., Koide, R.T., 2008. Can hypodermal passage cell distribution limit root penetration by mycorrhizal fungi? New Phytol. 180, 696–701.

Sokalska, D.I., Haman, D.Z., Szewczuk, A., Sobota, J., Deren, D., 2009. Spatial root distribution of mature apple trees under drip irrigation system. Agric. Water Manag. 96, 917–924.

Song, Y., Ye, L., Nii, N., 2011. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Hort. 129, 554–560.

Soukup, A., Malá, J., Hrubcová, M., Kálal, J., Votrubová, O., Cvikrová, M., 2004. Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry-tree plantlets during acclimation. Biol. Plant. 48, 481–489.

Steudle, E., Peterson, C.A., 1998. How does water get throught roots? J. Exp. Bot. 49, 775–788.

Storey, R., Jones, R.G.W., Schachtman, D.P., Treeby, M.T., 2003a. Calcium-accumulating cells in the meristematic region of grapevine root apices. Funct. Plant Biol. 30, 719–727.

Storey, R., Schachtman, D.P., Thomas, M.R., 2003b. Root structure and cellular chloride, sodium and potassium distribution in salinized grapevines. Plant Cell Environ. 26, 789–800.

Storey, R., Walker, R.R., 1987. Some effects of root anatomy on K, Na and Cl loading of citrus roots and leaves. J. Exp. Bot. 38, 1769–1780.

Stover, D.B., Day, F.P., Drake, B.G., Hinkle, C.R., 2010. The long-term effects of CO_2 enrichment on fine root productivity, mortality, and survivorship in a scrub-oak ecosystem at Kennedy Space Center, Florida, USA. Environ. Exp. Bot. 69, 214–222.

Sun, T., Mao, Z., 2011. Functional relationships between morphology and respiration of fine roots in two Chinese temperate tree species. Plant Soil 346, 375–384.

Sutton, R.F., 1980. Root system morphogenesis. New Zeal. J. For. Sci. 10, 264–292.

Taiz, L., Zeiger, E., Moller, I.M., Murphy, A., 2015. Plant physiology and development, 6th ed. Sinauer Associates, Inc., Sunderland, MA, USA.

Taleisnik, E., Peyrano, G., Cordoba, A., Arias, C., 1999. Water retention capacity in root segments differing in the degree of exodermis development. Ann. Bot. 83, 19–27.

Tawa, Y., Takeda, H., 2015. Which is the best indicator for distinguishing between fine roots with primary and secondary development in *Cryptomeria japonica* D. Don: Diameter,

branching order, or protoxylem groups? Plant Root 9, 79–84.

Taylor, J.H., Peterson, C.A., 2000. Morphometric analysis of *Pinus banksiana* Lamb. root anatomy during a 3-month field study. Trees 14, 239–247.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Thaler, P., Pagès, L., 1996. Root apical diameter and root elongation rate of rubber seedlings (*Hevea brasiliensis*) show parallel responses to photoassimilate availability. Physiol. Plant. 97, 365–371.

Thaler, P., Pagès, L., 1998. Modeling the influence of assimilate availability on root growth and architecture. Plant Soil 201, 307–320.

Tromp, J., 1975. The effect of temperature on growth and mineral nutrition of fruits of apple, with special reference to calcium. Physiol. Plant 33, 87–93.

Tromp, J., 1983. Nutrient reserves in roots of fruit trees, in particular carbohydrates and nitrogen. Plant Soil 71, 401–413.

Valenzuela-Estrada, L.R., Vera-Caraballo, V., Ruth, L.E., Eissenstat, D.M., 2008. Root anatomy, morphology, and longevity among root orders in *Vaccinium corymbosum* (*Ericaceae*). Am. J. Bot. 95, 1506–1514.

Van de Geijn, S.C., Petit, C.M., 1979. Transport of divalent cations. Cation exchange capacity of intact xylem vessels. Plant Physiol. 64, 954–958.

Van Geel, M., De Beenhouwer, M., Ceulemans, T., Caes, K., Ceustermans, A., Bylemans, D., Gomand, A., Lievens, B., Honnay, O., 2016. Application of slow-release phosphorus fertilizers increases arbuscular mycorrhizal fungal diversity in the roots of apple trees. Plant Soil 402, 291–301.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Vercambre, G., Pages, L., Doussan, C., Habib, R., 2003. Architectural analysis and synthesis

of the plum tree root system in an orchard using a quantitative modelling approach. Plant Soil 251, 1–11.

Volder, A., Smart, D.R., Bloom, A.J., Eissenstat, D.M., 2005. Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: Implications for root efficiency and competitive effectiveness. New Phytol. 165, 493–501.

Waisel, Y., Eshel, A., 2002. Functional diversity of various constituents of a single root sytem, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 157–174.

Walker, R.R., Sedgley, M., Blesing, M.A., Douglas, T.J., 1984. Anatomy, ultrastructure and assimilate concentrations of roots of citrus genotypes differing in ability for salt exclusion. J. Exp. Bot. 35, 1481–1494.

Wang, Z., Burch, W.H., Mou, P., Jones, R.H., Mitchell, R.J., 1995. Accuracy of visible and ultraviolet light for estimating live root proportions with minirhizotrons. Ecology 76, 2330–2334.

Wang, H., Inukai, Y., Yamauchi, A., 2006a. Root development and nutrient uptake. Crit. Rev. Plant Sci. 25, 279–301.

Wang, Z., Guo, D., Wang, X., Gu, J., Mei, L., 2006b. Fine root architecture, morphology, and biomass of different branch orders of two Chinese temperate tree species. Plant Soil 288, 155–171.

Wang, J., Zhang, X., Wu, C., 2015. Advances in experimental methods for root system architecture and root development. J. For. Res. 26, 23–32.

Weemstra, M., Mommer, L., Visser, E.J.W., van Ruijven, J., Kuyper, T.W., Mohren, G.M.J., Sterck, F.J., 2016. Towards a multidimensional root trait framework: A tree root review. New Phytol. 211, 1159–1169.

Weerdenburg, C.A., Peterson, C.A., 1983. Structural changes in phi thickenings during primary and secondary growth in roots. 1. Apple (*Pyrus malus*) *Rosaceae*. Can. J. Bot. 61, 2570–2576.

Wells, C.E., Eissenstat, D.M., 2001. Marked differences in survivorship among apple roots of different diameters. Ecology 82, 882–892.

Wells, C.E., Eissenstat, D.M., 2003. Beyond the roots of young seedlings: The influence of age

and order on fine root physiology. J. Plant Growth Regul. 21, 324–334.

Wells, C.E., Glenn, D.M., Eissenstat, D.M., 2002a. Changes in the risk of fine-root mortality with age: A case study in peach, *Prunus persica* (*Rosaceae*). Am. J. Bot. 89, 79–87.

Wells, C.E., Glenn, D.M., Eissenstat, D.M., 2002b. Soil insects alter fine root demography in peach (*Prunus persica*). Plant Cell Environ. 25, 431–439.

White, P.J., 1998. Calcium channels in the plasma membrane of root cells. Ann. Bot. 81, 173–183.

White, P.J., 2001. The pathways of calcium movement to the xylem. J. Exp. Bot. 52, 891–899.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

White, P.J., Piñeros, M., Tester, M., Ridout, M.S., 2000. Cation permeability and selectivity of a root plasma membrane calcium channel. J. Membr. Biol. 174, 71–83.

Wilcox, H., 1962. Growth studies of the root of incense cedar, *Libocedrus decurrens*. II. Morphological features of the root system and growth behavior. Am. J. Bot. 49, 237–245.

Wilcox, H.E., 1968a. Morphological studies of the root of red pine, *Pinus resinosa*. I. Growth characteristics and patterns of branching. Am. J. Bot. 55, 247–254.

Wilcox, H.E., 1968b. Morphological studies of the roots of red pine, *Pinus resinosa*. II. Fungal colonization of roots and the development of mycorrhizae. Am. J. Bot. 55, 688–700.

Wilsdorf, R., 2011. Evaluating the seasonal changes in calcium concentration and distribution in apple fruit after application of diferent calcium fertilisation strategies. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Withington, J.M., Reich, P.B., Oleksyn, J., Eissenstat, D.M., 2006. Comparisons of structure and life span in roots and leaves among temerate trees. Ecol. Monogr. 76, 381–397.

Xia, M., Guo, D., Pregitzer, K.S., 2010. Ephemeral root modules in *Fraxinus mandshurica*. New Phytol. 188, 1065–1074.

Yang, H., Jie, Y., 2005. Uptake and transport of calcium in plants. J. Plant Physiol. Mol. Biol. 31, 227–234.

Yang, H., Jie, Y., Shao, X., 2011. Regulation of calcium uptake and translocation in plants. Acta Hort. 903, 1011–1016.

Yao, S., Merwin, I.A., Brown, M.G., 2009. Apple root growth, turnover, and distribution under

different orchard groundcover management systems. HortScience 44, 168–175.

Yao, S., Merwin, I.A., Brown, M.G., 2006. Root dynamics of apple rootstocks in a replanted orchard. HortScience 41, 1149–1155.

Zadworny, M., Eissenstat, D.M., 2011. Contrasting the morphology, anatomy and fungal colonization of new pioneer and fibrous roots. New Phytol. 190, 213–221.

Zadworny, M., Mccormack, M.L., Rawlik, K., Jagodzin, A.M., 2015. Seasonal variation in chemistry, but not morphology, in roots of *Quercus robur* growing in different soil types. Tree Physiol. 35, 644–652.

Zimmerman, H.M., Steudle, E., 1998. Apoplastic transport across young maize roots: Effect of the exodermis. Planta 206, 7–19.

Zimmermann, H.M., Hartmann, K., Schreiber, L., Steudle, E., 2000. Chemical composition of apoplastic transport barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta 210, 302–311.

Table 1. Stepwise root sampling approach to separate fine roots that function primarily in nutrient absorption from those that function primarily in nutrient transport and storage.

	Fine root category	Root branch order	Action	Function
Step 1	Lifespan	First- and second-order roots		Ephemeral roots = absorptive
		Third-order roots upwards		Perennial roots = transport/storage
Step 2	Development	First-order roots	Separate fibrous and pioneer roots	Pioneer roots initially absorptive, but differ in both structure and function
		Second-order roots		
Step 3	Age	First-order fibrous roots	Separate white and brown roots	White and brown, first-order fibrous roots = absorptive
		Second-order fibrous roots		White, second-order fibrous roots = absorptive
Step 4	Age	Brown, second-order fibrous roots	Separate primary and secondary developed roots	Primary developed, brown, second-order fibrous roots = absorptive
				Secondary developed, brown, second-order fibrous roots = transport/storage

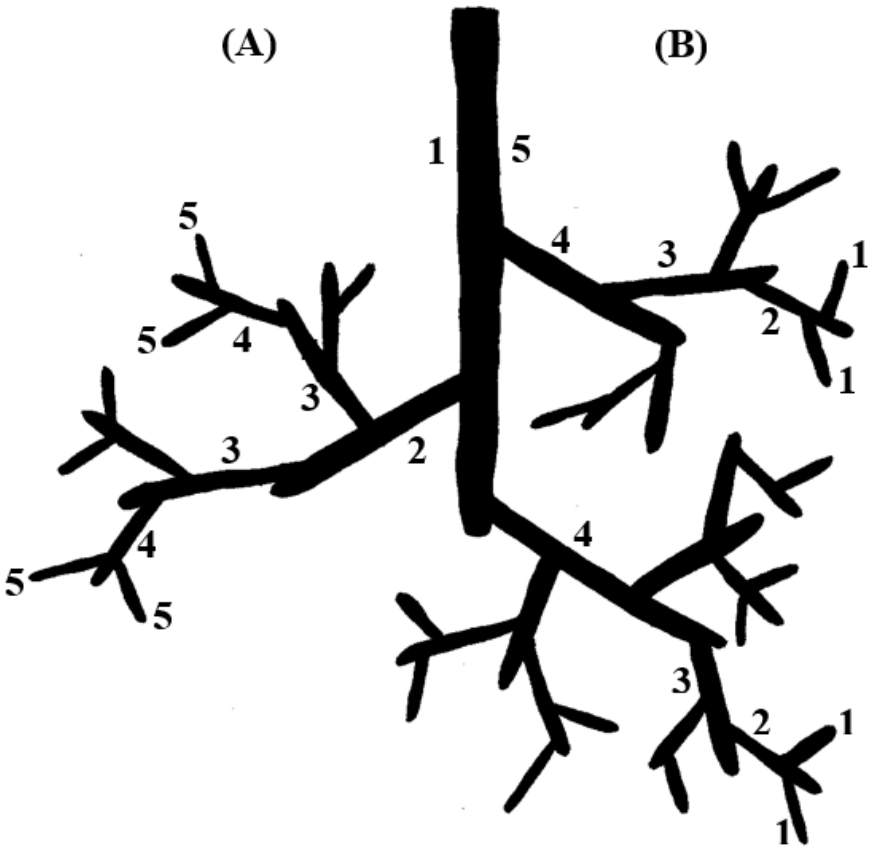


Fig. 1. A schematic representation of an intact branch root consisting of five root orders, either classified according to the sequence of lateral root emergence (scheme 1: A), or root hierarchal position (scheme 2: B)

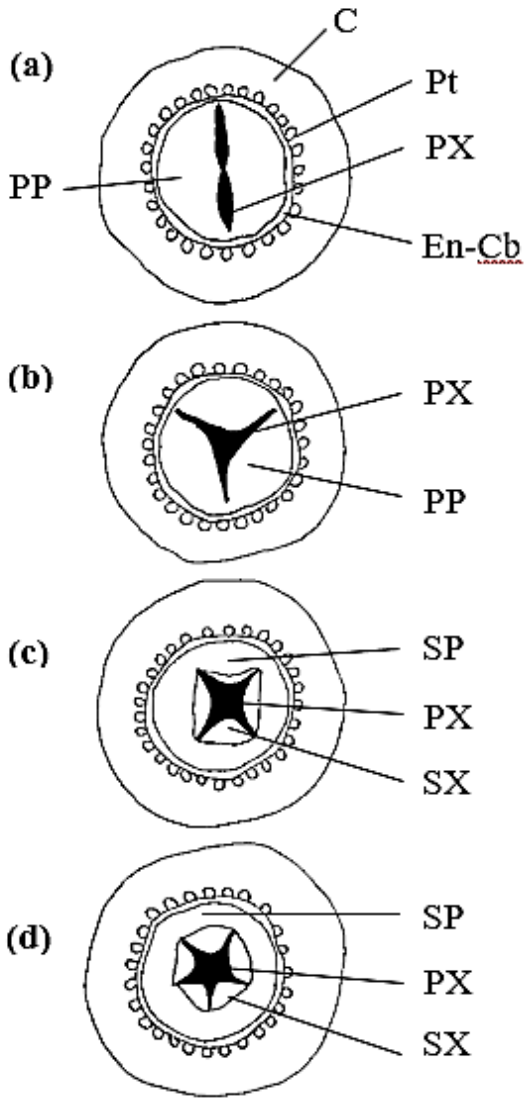


Fig. 2. Representation of the internal vascular pattern of roots. (a) Diarch root with primary development. (b) Triarch root with primary development. (c) Tetrarch root with secondary development. (d) Poly-arch root with secondary development. C, cortex; Pt, phi thickenings; PP, primary phloem; PX, primary xylem; En-Cb, endodermis with Casparian bands; SP, secondary phloem; SX, secondary xylem.

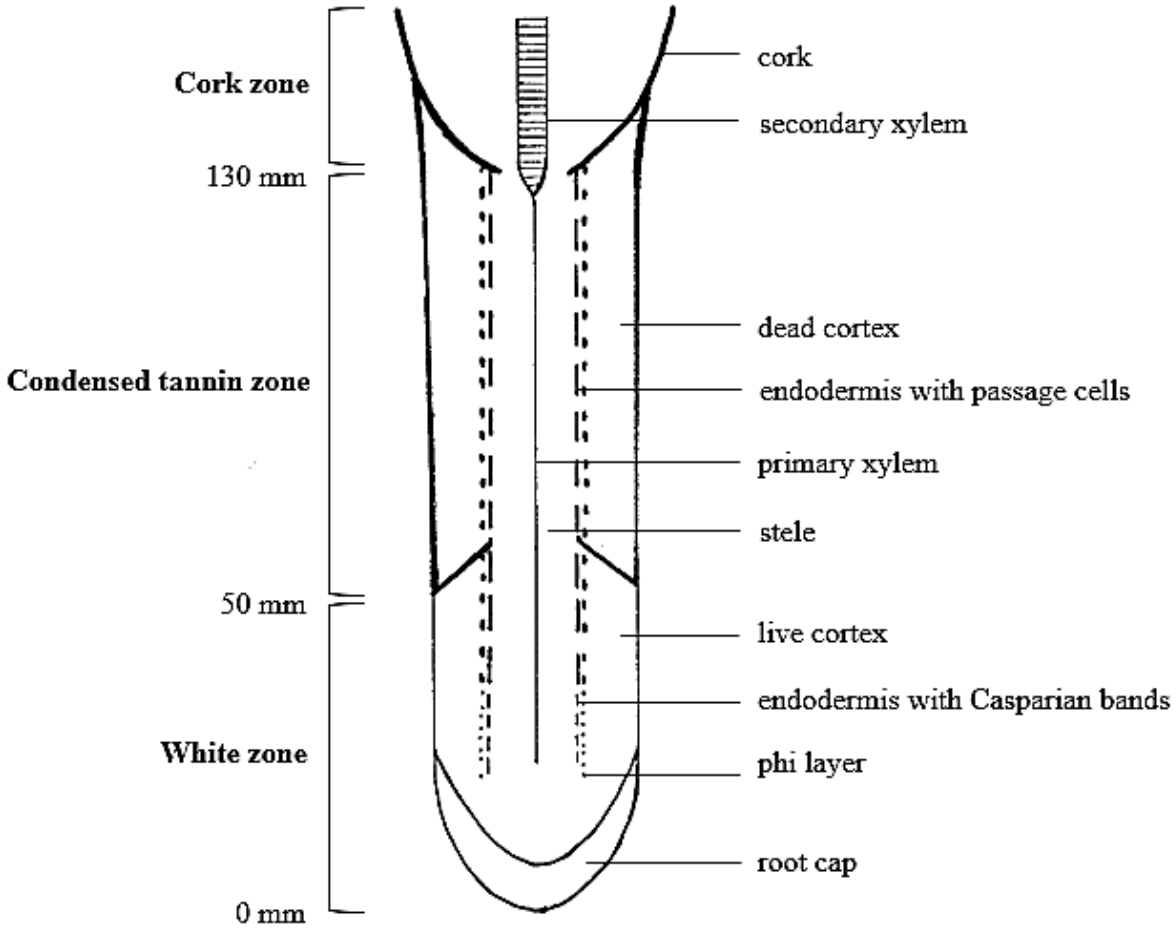


Fig. 3. Representation of the three anatomically distinct zones in a woody apple root, illustrating the locations of various anatomical features.

PAPER 1

Impact of soil-applied calcium nitrate and autumn defoliation on root growth and calcium uptake and partitioning in young, potted apple trees during winter

Abstract

To optimize Ca reserve accumulation in the roots and permanent structural components of deciduous fruit trees under local conditions of insufficient winter chilling, knowledge of the dynamics of Ca uptake by roots and its allocation during autumn and into winter is required. Young, non-bearing 'Golden Delicious' apple trees (*Malus domestica* Borkh.) on M7 rootstock were potted in 25 L plastic containers and fertigated with a low calcium (Ca), balanced nutrient solution from planting (September 2015) until the end of winter (August 2016). To assess the uptake and partitioning of Ca, as effected by level of soil Ca supply, additional applications of calcium nitrate ($Ca(NO_3)_2$) were supplied to the soil in May 2016, towards the end of autumn and into winter, to trees that experienced natural leaf drop (NLD). Calcium was applied at three different rates: no additional Ca (control), moderate Ca (75 g $Ca(NO_3)_2$ pot^{-1}) and high Ca (150 g $Ca(NO_3)_2$ pot^{-1}). Soil applications were synchronized with a period of active white root growth. To assess the impact of early leaf loss on Ca uptake and reserve accumulation during winter, another group of 2X treated trees were hand-defoliated earlier in autumn, during April 2016. Additionally, to monitor sap flow dynamics in relation to Ca uptake and distribution throughout the trees during winter, single dendrometers were installed in April 2016 on both NLD and autumn defoliated (AD) trees that received the high Ca treatment. All trees were destructively sampled at the end of August 2016 for Ca and nitrogen (N) analysis. At the expense of Ca reserve accumulation in the roots and stems, a significant higher leaf Ca concentration was observed in the $2X_{(NLD)}$ treatment. An increase in soil Ca supply did not have an impact on white root growth in the NLD treatments. In contrast, root Ca uptake and reserve accumulation in the $2X_{(AD)}$ treatment was not compromised, despite a significant decrease in root dry mass, together with a steep decline in white root numbers within two weeks of treatment. Root Ca concentration of the $2X_{(AD)}$ treatment was significantly higher compared to the $2X_{(NLD)}$ treatment, whereas no significant differences in stem Ca concentration were found. No difference in maximum daily shrinkage of stems (MDS) between the $2X_{(NLD)}$ and

$2X_{(AD)}$ treatments was detected after 50 % leaf drop (July 2016). However, MDS remained above zero until the end of winter. This provided evidence that active sap flow (xylem and phloem) still occurred after 50 % leaf drop despite the decline in transpiration in the NLD treatment. In addition, despite the absence of leaves, active sap flow in the AD treatment indicated that leaf transpiration-driven sap flow is not the main driver for root Ca uptake and translocation in the xylem of young, non-bearing apple trees during dormancy.

Keywords: Dendrometer, maximum daily stem shrinkage, sap flow, transpiration, white roots.

1. Introduction

It is well known that carbohydrate and nutrient reserves, accumulated in the roots and stems of deciduous fruit crops at the end of the previous growing season, are remobilized to support growth and metabolism of newly developing tissues in spring, when current photosynthates and nutrient supplies are lacking (Ferguson, 1980; Tromp, 1983; Oliveira and Priestley, 1988; Millard and Grelet, 2010). In fact, as high as 50 to 80 % of the total non-structural carbohydrate (TNC) and N reserve content in the roots and stems of fruit trees may be remobilized in spring (Lacointe et al., 1993; Neilsen et al., 2001; Guak et al., 2003; Cheng and Raba, 2009; Roccuzzo et al., 2017). In apple, stored carbohydrates that are mainly composed of starch, soluble sugars (sucrose, glucose and fructose) and sorbitol (Yoshioka et al. 1988), are primarily utilized for respiration, rather than for growth (Kandiah, 1979; Loescher et al., 1990). Before the shoots of apple trees become self-sufficient at approximately 15 to 19 days after bud break (Oliveira and Priestley, 1988), less than 20 % of the carbon (C) required for new growth is supplied by reserve carbohydrates (Kandiah, 1979). Indeed, Cheng and Fuchigami (2002) reported that the initial growth of young apple trees in spring is more dependent on reserve N than reserve carbohydrates and that the latter are mainly utilized to provide C skeletons and energy for N assimilation.

Higher plants that receive adequate amounts of Ca from their natural environment generally contain between 1 – 50 mg Ca g^{-1} dry mass (Kirkby and Pilbeam, 1984). Previous studies on apple (Terblanche, 1972; Terblanche et al., 1979; Kangueehi, 2008), kiwifruit (Ferguson and Turner, 1981; Kotzé and De Villiers, 1989) and grape (Conradie, 1981) indicated major contributions of the roots and permanent structural components to the Ca content of new growth of leaves, shoots and fruit early in spring. Terblanche (1972) found that 49 % of the Ca

content in the new growth of young, potted apple trees was supplied by the reserves in the roots and wood during the first 22 days after bud break. Furthermore, approximately one third of the Ca content in the new growth originated from the reserves in the bark following this period until extension growth of 50 % of the shoots was complete. As Ca destined for the shoot is not directly transported from the bark, the latter supply possibly came from the wood following remobilization and radial transport of available Ca (Ferguson, 1980; Ferguson and Turner, 1981). In agreement, Kangueehi (2008) reported that almost 70 % of the Ca content in the new growth of young, bearing apple trees in the field was supplied by reserves located in the permanent parts of the trees during the first six weeks after bud break. Limited uptake of Ca from the soil occurred during this period, possibly because young white roots or root tips, that are generally considered to have the highest potential for soil nutrient uptake (Clarkson, 1984, 1993; Bouma et al., 2001; Eissenstat et al., 2001; Volder et al., 2005; De Freitas and Mitcham, 2012; Gu et al., 2015), are mostly absent during spring in locally grown apple trees (Van Zyl, 2016).

Van Zyl (2016) established that young non-bearing apple trees have the potential to continuously produce new white roots at some level throughout the growing season, while root production in bearing apple trees follows a more prominent bimodal pattern. An initial minor flush in summer (November/December) is usually followed by a second, more prominent flush in autumn and into winter (March to August). Numerous studies have shown that no single factor exclusively controls the timing and magnitude of root growth flushes in deciduous trees. It is rather influenced by a complex interaction of combinations of environmental factors, endogenous tree factors, as well as fixed phenological growth events that involve a complex system of source-sink interactions (Kozlowski, 1992; Côté et al., 1998; Joslin et al., 2001; Anderson et al., 2003; Gaudinski et al., 2009; Montagnoli et al., 2014; Abramoff and Finzi, 2015; Contador et al., 2015). This dynamic was well demonstrated by Van Zyl (2016) in various apple orchards in the warmer growing regions of the Western Cape. Where soil temperature and moisture levels proved non-limiting to root growth throughout the year, it was concluded that the temporal pattern of white root production in apple under local growing conditions may be ascribed to endogenous control factors, e.g. the availability of current assimilates from photosynthesis. Similar findings have been reported for apple trees under Mediterranean-type climatic conditions in Western Australia (Cripps, 1970). Additionally, in various plant species, treatments such as defoliation, canopy scorching or branch pruning have been shown to adversely affect new root production through reductions in below-ground C

availability (Maggs, 1965; Head, 1967, 1969; Atkinson, 1972; Guo et al., 2004; Kwack et al., 2014), while treatments such as an increase in soil Ca supply have been shown to stimulate lateral root initiation and growth (Emanuelsson, 1984; Poovaiah and Reddy, 1991).

Calcium absorbed by the roots proceeds upwards in the xylem sap not primarily by transpiration-driven mass flow, but via a series of reversible exchange reactions across a fixed number of non-specific ion exchange sites situated on and in the walls of the lignified xylem column (Shear and Faust, 1970; Clarkson, 1984; Atkinson et al., 1992; Gilliham et al., 2011). The rate of Ca ascent depends not only on the rate of transpiration, but also on the solution concentration, whether Ca is chelated or not, the number and loading status of the exchange sites in the xylem column and the rate of ion removal from these sites (Biddulph et al., 1961; Bell and Biddulph, 1963; Ferguson and Bollard, 1976; Hanger, 1979; Van de Geijn and Petit, 1979). While enhanced transpiration may accelerate nutrient uptake from the soil (Tromp, 1975; Kirkby, 1979; Gilliham et al., 2011), Ca^{2+} translocation rates are strongly associated with the metabolic demands of the respective plant parts (Biddulph et al., 1961; Bell and Biddulph, 1963; Engels 1999). Root Ca uptake may either proceed via the apoplastic pathway (extracellular) which is relatively non-selective and more dependent on transpiration, or via the symplastic pathway (inter- and intracellular) which is more selective, with less dependency on transpiration, allowing roots to control the rate of Ca movement into the xylem based on metabolic demand (White, 1998, 2001; White and Broadley, 2003; Wang et al., 2006). Once Ca^{2+} reaches the xylem, the rate of transpirational water flow merely determines the distance Ca^{2+} travels between successive deposition with exchange reactions along the exchange sites in the xylem column (Del Amor and Marcelis, 2006). Calcium is not only translocated in the xylem in its ionic form. A marked percentage of the total Ca content in the xylem sap may be translocated as uncharged or negatively charged complexes with organic acids, mainly malic- and citric acids (Prima-Putra and Botton, 1998). Transport in this non-cationic form can increase the mobility of Ca in the xylem by reducing its retention on the exchange sites (Bradfield, 1976). From the onset of leaf drop, a substantial amount of Ca may be distributed to the roots and stems of apple trees following soil fertilization (Terblanche, 1972; Terblanche et al., 1979; Van Zyl, 2016), suggesting that leaf transpiration is not the only factor governing the upward movement of Ca in the xylem sap of trees (Tanner and Beevers, 2001; Montanaro et al., 2010; Singh, 2016). Under conditions of insufficient winter chilling in the warmer apple growing regions of the Western Cape (Midgley and Lötze, 2011), young trees are known to undergo an extended leaf drop period (personal communication, Dr. E. Lötze, Department of

Horticultural Science, Stellenbosch University). In agreement, Heide and Prestrud (2005) found that growth cessation, leaf senescence and abscission and dormancy induction in apple and pear trees only occur in response to low temperature (< 12 °C). Yet, no studies on the influence of leaf drop on Ca uptake and partitioning in relation to sap flow dynamics in apple trees during winter in South Africa could be sourced.

Since reserve Ca stored in the roots, wood and bark of apple trees is the main source of Ca supply for new growth in spring, an effective strategy to ensure a maximum supply of Ca to the leaves, shoots and fruit early in the next season could be to optimize the replenishment of Ca reserves in the trees during autumn and with the onset of winter (Terblanche et al., 1979). Del Amor and Marcelis (2003) reported a strong correlation between root growth activity and plant Ca concentration. In young, non-bearing 'Golden Delicious'/M7 apple trees potted in 5 L plastic containers (Van Zyl, 2016), an industry recommended standard rate of calcium nitrate (8 g $Ca(NO_3)_2$ pot^{-1}) applied to the soil at the beginning of April (mid-autumn) proved sufficient to significantly increase the Ca concentration of the roots and stems, three weeks after application. Therefore, a next strategy which may become a standard practice in local commercial orchards to ensure the optimization of Ca reserves in apple trees may be accomplished by synchronizing additional soil Ca applications with periods of active white root growth as it is known to occur under South African conditions during autumn. Thus, to address the effect of dormancy on Ca uptake and partitioning in apple trees, the current study was designed to examine the effect of synchronized high $Ca(NO_3)_2$ soil applications (i.e. 1.5 × standard application rate and 4 × standard application rate) with active white root growth at the end of autumn on Ca accumulation in the different tissues of young, potted, non-bearing apple trees at the end of winter.

The objectives of this study were: (1) to quantify whether additional soil $Ca(NO_3)_2$ applications towards the end of autumn and into winter during active white root growth can contribute substantially to Ca reserve accumulation in the roots and permanent structural components of the tree, (2) to determine whether an increase in soil $Ca(NO_3)_2$ supply has an impact on white root growth, (3) to determine whether extended leaf drop under local growing conditions (insufficient winter chilling) influences Ca reserve accumulation in the tree, and (4) to quantify the impact of leaf drop on white root growth in young, potted, non-bearing apple trees during autumn and into winter. Even though the study focused on the dynamics of Ca uptake and partitioning, the trees also received an increased supply of nitrate (NO_3^-) in the form of

$Ca(NO_3)_2$. Due to the promotive effects of NO_3^--N on vegetative growth as well as Ca^{2+} uptake and translocation in apple trees, especially to the older leaves (Shear and Faust, 1970; Korcak, 1980; Wallace and Mueller, 1980), N distribution following additional $Ca(NO_3)_2$ applications was also considered.

2. Materials and Methods

2.1. Plant material and experimental design

The study was conducted on the Welgevallen Experimental Farm (33°56'33"S, 18°51'56"E) of Stellenbosch University in the Western Cape, South Africa. Dormant, two-year-old 'Golden Delicious' apple trees on M7 rootstock from Stargrow Nursery (Stellenbosch, SA) were subjected to a cold treatment of 4 °C for one month before planting to ensure synchronized budding in spring. At planting, each tree was headed 10 cm below the apex to encourage lateral shoot growth and development.

Trees were individually potted in 35 cm diameter (25 L) brown plastic containers filled with a 4:1 (*v/v*) mixture of coarse sand (Pool Filter Sand, Builders Express, Stellenbosch) and compost (Boutique 20DM, Builders Express, Stellenbosch). A single, composite sample of the growing medium from two randomly selected pots at planting was analysed by a commercial laboratory (Bemlab (Pty) Ltd., 16 Van der Berg Crescent, Strand, South Africa, 7140) to determine the Ca concentration (cmol(+) kg^{-1}) of the growing medium (Annexure 1, Table 1). Pots were placed on a transparent plastic tarp, evenly spaced in rows, with an additional sheet of white plastic tarp strapped around the base and sides of the pots along each individual row to reflect sunlight and reduce heat build-up in the pots (Fig. 1). When the maximum temperature of the growing medium increased to > 35 °C in January 2016, a thin mulch, consisting of a 1 – 2 cm thick layer of bark chips, was applied to cover the surface of each pot to buffer the temperature of the growing medium.

Trees were grown outdoors in full sunlight from end of September 2015 to end of August 2016, where they were fertigated daily with a balanced nutrient solution through an automatically controlled drip irrigation system. Two Netafim (www.netafim.co.za) drippers (2 L h^{-1}) were spaced on opposite ends of the tree stem in each pot. Fertigation was set at 4 min cycles four times per day (0.5 L day^{-1} pot^{-1}) up to early summer (15 December 2015), after which it was

increased to 4 min cycles six times per day (0.8 L day^{-1} pot^{-1}) as the growing medium tended to dry out. The nutrient solution was custom prepared and had the following composition: KNO_3 at 757.5 mg L^{-1}; KH_2PO_4 at 102 mg L^{-1}; $MgSO_4.7H_2O$ at 645.75 mg L^{-1}; $Ca(NO_3)_2.2H_2O$ at 150 mg L^{-1} (personal communication, Dr. E. Kempen, Department of Agronomy, Stellenbosch University), with a final EC of 1.5 mS cm^{-1}. Just the minimum Ca requirement for growth was met to allow for a response to additional Ca treatments.

The experimental layout was a randomized complete block design with four treatments and 12 blocks, with single tree replicates as experimental units. The treatments comprised soil applications of $Ca(NO_3)_2$ (YaraLiva Nitrabor®, Yara Africa Fertilizer (Pty). Ltd.) applied at two different rates: moderate (1X) and high (2X). The 1X treatment consisted of 75 g $Ca(NO_3)_2$ granules pot^{-1} and the 2X treatment of 150 g $Ca(NO_3)_2$ granules pot^{-1}. The 2X treatment was applied to trees that experienced natural leaf drop throughout autumn and to trees that were subjected to complete manual defoliation on 22 April 2016. The Ca treatments, applied by hand, were split into equal quantities on a weekly basis, over a three-week period. Application dates were as follows: 6, 13 and 20 May 2016. To ensure that the granules were dissolved, each pot was watered with approximately 2 L of tap water after each application. Control trees received no additional $Ca(NO_3)_2$ applications. The timing of soil applications were based on previous reports on white root growth dynamics of bearing apple trees in the Western Cape (Van Zyl, 2016). The control, 1X treatment and 2X treatment that experienced natural leaf drop (NLD), will be referred to as "control", "1X" and "$2X_{(NLD)}$", and the 2X treatment that was subjected to complete autumn defoliation (AD), as "$2X_{(AD)}$".

2.2. Tree phenology

To relate the pattern of white root growth to above-ground tree phenology during autumn/winter, the following phenological growth stages were visually determined: shoot growth cessation, onset of leaf drop, 50 % leaf drop and 100 % leaf drop.

2.3. Measurements

2.3.1. Soil temperature

Individual Tiny-tag soil probes (TPG-4505 Gemini Data Loggers Ltd., Chichester, West Sussex, UK) were inserted at a depth of 15 cm below the surface of the growing medium in two randomly selected pots to record hourly soil temperatures (°C) for the duration of the trial.

2.3.2. White root growth

To monitor white root growth of the control, 1X, $2X_{(NLD)}$ and $2X_{(AD)}$ treatments, eight clear acrylic butyrate tubes (minirhizotrons) were installed in randomly selected pots at the time of planting (end of September 2015). One tube was installed per tree to represent one of two replications per treatment. Each tube (80 mm diameter x 0.5 m length) was inserted at a 45° angle from the vertical, parallel to the work row, with the base slanted away from the stem. A white polyethylene container was placed over the exposed end of each tube protruding from the surface of the growing medium to prevent light penetration and radiant heating in the root zone. Root scanning commenced three months after installation to allow the growing medium to settle and natural root growth to occur (Fig. 1).

High resolution (300 DPI) 360° digital colour images (21.6 x 19.6 cm window) were recorded with a CI-600 In-Situ Root Imager (CID Bioscience, Camas, WA, USA). Root scans were performed on a bi-weekly basis (weather permitting). White root growth was quantified by manually counting the number of white root tips in each sequential digital image of the root zone (Fig. 2). The average white root count per treatment was plotted against observation dates for the period January to August 2016 to present white root growth patterns before and after treatment.

2.3.3. Tree height and stem diameter

Tree height and stem diameter measurements were recorded at both the start (September 2015) and end of the trial (August 2016). Tree height (m) was measured with a measuring tape. Stem diameter (mm) was measured at 2 cm above the graft union with a digital Vernier calliper.

2.3.4. Nutrient distribution

To obtain base line data representing a typical dormant tree at planting, 12 additional trees were randomly selected and individually separated into their component parts at planting for Ca analysis. To quantify the effect of increasing levels of soil-applied $Ca(NO_3)_2$ on the distribution of Ca and N in the tree towards the end of winter, the control, 1X treatment and $2X_{(NLD)}$ treatment were considered. All the abscised leaves of the $2X_{(NLD)}$ treatment were recovered from light-weight nets (Netlon Netting, SA) that were draped over the tree canopies and secured with fishing line at the base around their main stems, before NLD started (Fig. 3). Although the net shading percentage is unknown, an open net structure was selected to limit shading of the leaves that could potentially affect photosynthesis.

To evaluate the effect of AD on the distribution of Ca and N in the tree at the end of winter, only the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments were considered. Upon completion of AD, the fresh mass (FM) of all the leaf samples was recorded, then oven-dried at 70 °C to a constant mass for dry mass (DM) measurements.

At the end of August 2016, all the trees were excavated and individually separated into their component parts for Ca and N analysis. Roots were soaked in water until it was possible to separate them from the potting medium with minimum breakage, then washed with water to remove any adhering particles. The FM and DM of all the samples were recorded. To compare the Ca concentration of the roots and stems at planting to that at the end of winter, dried tissue samples of eight single tree replicates were randomly selected for destructive mineral analysis. All mineral analyses were carried out by Bemlab (Pty). Ltd. (16 Van der Berg Crescent, Strand, South Africa, 7140). Calcium and N concentrations are expressed as % DM. The concentrations of phosphorus (P), potassium (K), magnesium (Mg) and sodium (Na) were not considered in this study, as these elements were applied equally as a balanced nutrient application to all treatments. The Ca and N content (g DM), respectively, per plant part was determined by multiplying the concentration of each respective element (% DM) with the corresponding DM (g) of each plant part (Annexure 1, Table 2). To determine the percentage distribution of Ca and N, respectively, in each plant part (% of total), the amount of each nutrient element (g DM) per plant part was divided by the total content of each nutrient element (g DM) per tree (Annexure 1, Table 2) and expressed as a percentage.

2.3.5. Sap flow dynamics

Electronic dendrometers (Model DEX70, Dynamax Inc., Houston, TX, USA) were used to continuously monitor diurnal stem diameter changes over time according to procedures described by Link et al. (1998). On 14 April 2016, single dendrometers were installed around the main stems, approximately 5 cm above the graft union, of three single tree replicates per treatment. Treatments included the $2X_{(NLD)}$ treatment and $2X_{(AD)}$ treatment (Fig. 4).

Dendrometer readings were recorded hourly for the duration of the trial using a CR1000 data logger (Campbell Scientific, Logan, UT). The linear calibration equation for each individual dendrometer (source: Dynamax Inc., Houston, TX, USA) was used to convert the millivolt (mV) output to mm units.

The use of DEX70 dendrometers to measure thickness dynamics of apple tree trunks and fruit in the field showed that changes in ambient temperature can affect the dendrometer accuracy and may cause up to 10 % error in readings if not accounted for (Link et al., 1998). To compensate for the effect of expansion and contraction in the steel arm of the dendrometer due to fluctuations in ambient temperature, a single dendrometer was installed around a 20 mm diameter PVC cylinder that was secured in an upright position at the site. Hourly data was acquired over a two-week period and converted to mm units as described above. The effect of temperature on the sensor signal after conversion to mm units was determined using a linear regression as:

$$\text{mm} = b_0 + b_1 \times \text{°C}$$

In this equation, b_0 is the intercept value (mm) when the temperature is 0 °C, and b_1 is the slope of the relationship between diameter and temperature. The equation was used to predict the effect of temperature on each recorded mm unit (Link et al., 1998). To eliminate the effect of temperature, the deflection value (mm) was subtracted from each observation. For each mm unit to start at zero, the initial mm value of each dendrometer was subtracted from all subsequent values (Link et al., 1998) after which the hourly means per treatment were calculated. The maximum daily stem shrinkage (MDS) was calculated as the difference between the maximum stem diameter and the minimum stem diameter recorded per day (De Swaef et al., 2009). To evaluate the difference in sap flow dynamics between treatments, the average MDS values (mm) were plotted over time for the period April to August 2016.

2.4. Statistical analysis

In all analyses, the dependent variables were $\log_{10}$-transformed to achieve normality and homoscedasticity of variances among treatments where required. Tree height (m) and stem diameter (mm) were analysed with analysis of covariance (ANCOVA) using the general linear model (GLM) procedure. Means were separated using Least Square Means (LSM) test. To evaluate the effect of increasing levels of soil-applied $Ca(NO_3)_2$ on vegetative growth and nutrient distribution, a two-way analysis of variance (ANOVA) was performed using the GLM procedure. Means were separated using Fisher's LSD (ordinary pairwise *t*-tests). All data effects were considered statistically significant if $P \leq 0.05$. To compensate for temperature effects on the dendrometers, a linear regression analysis was used to model the relationship between ambient temperature (°C) and dendrometer deflection (mm). All statistical analyses were performed with Statistical Analysis System (SAS) version 9.4 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Tree phenology

In most trees, shoot growth cessation was noted mid-February. However, all trees of the control, IX treatment and $2X_{(NLD)}$ treatment showed a new growth flush to some extent from March to May (Fig. 5a). Unusually high ambient temperatures (23 °C to 28 °C; data not shown) were experienced during this period. Following the onset of leaf drop mid-May, a substantial number of healthy intact leaves were still present on most trees until 50 % leaf drop was recorded in July (Fig. 5b). Upon termination of the experiment at the end of August, trees reached approximately 90 % leaf drop with some leaves still firmly attached (Fig. 5c). In the $2X_{(AD)}$ treatment, several terminal buds burst in May (Fig. 5d). The developing buds were continuously pinched to prevent further growth.

3.2. White root growth

Root scans revealed new white root growth in all treatments throughout the season (Fig 6). Large variations in white root numbers were evident between replications throughout the trial period, indicating variation in pots. The large tree-to-tree variation made true comparisons between treatments difficult, but a trend was nonetheless observed. Prior to any treatments,

white root growth during summer (January/February) and into autumn (March/April) was inconsistent. A slow but steady increase in white root numbers were observed up to mid-May, during which time soil applications were executed. Following the onset of leaf drop mid-May, trees of the control, 1X treatment and $2X_{(NLD)}$ treatment showed a gradual decline in white root numbers as roots proceeded to mature during winter (June to July). In contrast, in the $2X_{(AD)}$ treatment, a sharp decline in white root numbers were observed within two weeks of defoliation, during May. Around mid-June to early-July, white root numbers were lower in the $2X_{(AD)}$ treatment compared to the NLD treatments. While white root numbers of the $2X_{(AD)}$ treatment remained relatively low for the rest of winter, white root numbers of the NLD treatments increased again from mid-July and remained constant up to the end of August.

3.3. Soil temperature

Since the temperatures recorded between the two pots did not differ, average values were calculated and used to plot soil temperature (°C) against time (months) (Fig. 7). Soil temperatures were high in January and February and decreased towards August. The maximum temperature recorded 15 cm below the surface was 42 °C in January and the minimum temperature, 3 °C in July.

3.4. Tree height and stem diameter

There were no significant differences in stem diameter or tree height between treatments or the control at the end of the trial (Table 1).

3.5. Vegetative growth

No significant differences in $FM_{(stems)}$ or $DM_{(stems)}$ were found between treatments, but significant differences in FM and DM of the roots, shoots and leaves were found (Table 2). With an increase in $Ca(NO_3)_2$ applications, a linear decrease in $FM_{(roots)}$ and $DM_{(roots)}$ was observed. The lowest $FM_{(roots)}$ and $DM_{(roots)}$ was found in the $2X_{(AD)}$ treatment and the $FM_{(roots)}$ and $DM_{(roots)}$ of the $2X_{(NLD)}$ treatment was significantly lower than the control. Although the $FM_{(roots)}$ and $DM_{(roots)}$ of the 1X treatment was lower than the control, this was not significant. In the NLD trees, increasing $Ca(NO_3)_2$ applications had no significant effect on biomass accumulation in the shoots, whereas the $2X_{(AD)}$ treatment caused a significant decrease in $FM_{(shoots)}$ and $DM_{(shoots)}$. Due to the experimental design, leaf data did not provide reasonable

evidence to support an effect of increasing $Ca(NO_3)_2$ applications on the $FM_{(leaves)}$ between treatments, but it did allow for a comparison between the $DM_{(leaves)}$ of the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments. The $DM_{(leaves)}$ of the $2X_{(AD)}$ treatment was significantly higher compared to the $2X_{(NLD)}$ treatment.

3.6. Nutrient distribution

3.6.1. Calcium

Despite an increase in soil $Ca(NO_3)_2$ supply, the Ca reserve status of the NLD trees remained unchanged. No significant differences were found between the Ca concentration of the roots and stems of the control at planting and the control, IX and $2X_{(NLD)}$ treatments at the end of the trial (Table 3). Defoliation prior to the commencement of soil applications, however, had a significant impact on the Ca concentration of the roots of the $2X_{(AD)}$ treatment, but not the stems. Compared to the NLD treatments, the $2X_{(AD)}$ treatment had the highest root Ca concentration, whereas no significant differences in stem Ca concentration were found (Table 3). Regarding the new growth, no significant differences in shoot Ca concentration were found between treatments, but the Ca concentration of the leaves differed significantly between treatments (Table 3). The highest Ca concentration was found in the leaves of the $2X_{(NLD)}$ treatment, while that of the control and 1X treatment did not differ significantly. Leaf Ca concentration of the $2X_{(AD)}$ treatment was significantly lower than the control and 1X and $2X_{(NLD)}$ treatments.

Within tree partitioning of Ca content (% of total) between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments are depicted in Fig. 8. As the total Ca content (g DM) as well as the % of total Ca content in each respective plant part was calculated on a DM basis, a comparison could only be made between these two treatments. At the end of the trial, the total Ca content (g DM) per tree did not differ significantly between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments (1.44 g DM vs 1.15 g DM) (Annexure 1, Table 2), but significant differences in % of total Ca content in the roots, stems, shoots and leaves were found. A higher % of total Ca content was found in the shoots (13 % vs 6 %) and leaves (37 % vs 31 %) of the $2X_{(NLD)}$ treatment, whereas a higher % of total Ca content was found in the roots (25 % vs 20 %) and stems (38 % vs 29 %) of the $2X_{(AD)}$ treatment.

3.6.2. Nitrogen

Significant differences in N concentration were found in the roots, stems, shoots and leaves between treatments (Table 3). With an increase in $Ca(NO_3)_2$ soil applications, root N concentration of the NLD trees increased linearly, with the highest N concentration found in the roots of the $2X_{(NLD)}$ treatment. Root N concentration of the $2X_{(AD)}$ treatment was significantly lower compared to the $2X_{(NLD)}$ treatment but did not differ significantly from the 1X treatment and was significantly higher compared to the control. The N concentration of the stems and shoots did not differ significantly between the 1X and $2X_{(NLD)}$ treatments but were significantly higher compared to the control and $2X_{(AD)}$ treatment, which did not differ significantly. Leaf N concentration of the 1X, $2X_{(NLD)}$ and $2X_{(AD)}$ treatments did not differ significantly but were significantly higher compared to the control.

Total N content (g DM) and within tree partitioning of N content (% of total) between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments are indicated in Annexure 1, Table 2 and Fig. 9, respectively. At the end of the trial, the total N content (g DM) per tree in the $2X_{(NLD)}$ treatment was significantly higher compared to the $2X_{(AD)}$ treatment (5.28 g DM vs 3.20 g DM). Although no significant differences in % of total N were detected in the stems between these two treatments, significant differences in the roots, shoots and leaves were found. A higher % of total N content was found in the roots (42 % vs 35 %) and shoots (11 % vs 4 %) of the $2X_{(NLD)}$ treatment, whereas a higher % of total N content was found in the leaves (24 % vs 11 %) of $2X_{(AD)}$ treatment.

3.7. Sap flow dynamics

The relationship between ambient temperature and DEX70 dendrometer deflections was determined by linear regression analysis. The relationship was found to be linear and highly significant (Fig. 10). By applying the linear equation (Fig. 10), the temperature effect on the dendrometers could be compensated for, and data could, therefore, be expressed for a constant temperature of 12 °C during autumn/winter.

In Fig. 11, the average MDS between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments is shown from mid- to end of April (before and after defoliation took place on 22 April), mid- to end of May (start of leaf drop), beginning to mid-July (50 % leaf drop) and beginning to mid-August (90 % leaf drop). Large variations between replicates were evident, but a trend was still observed. The MDS between treatments was similar up to 22 April (Fig. 11a). Following defoliation, MDS

of the $2X_{(NLD)}$ treatment was slightly higher compared to the $2X_{(AD)}$ treatment. With the onset of leaf drop mid-May, the difference in MDS between treatments remained relatively constant (Fig. 11b). After 50 % leaf drop, the difference in MDS between treatments became progressively smaller (Fig. 11c). There was no noticeable difference in MDS between treatments towards the end of winter when leaf drop approached 90 % (Fig. 11d).

4. Discussion

4.1. Tree vigour

Tree growth in terms of an increase in height and stem diameter was uniform among treatments. However, in trees that were left to drop their leaves naturally during autumn and into winter, terminal buds on the current season's growth flushed between March and May (personal observation) giving rise to late-season "lammas shoots" (Kozlowski, 1964; Codesido and Fernandez-Lopez, 2009; Beikircher and Mayr, 2013). Raese and Staiff (1990) showed that high rates of soil-applied $Ca(NO_3)_2$ (2.1 kg $tree^{-1}$) in spring increased tree vigour in young bearing 'Delicious' and 'Golden Delicious' apple trees. This was attributed to high rates of N fertilization during three seasons. In the present study, however, no significant differences in shoot mass were found between the 1X treatment or $2X_{(NLD)}$ treatment and the control. The highest N rate applied (0.15 kg $Ca(NO_3)_2$ $tree^{-1}$) was much lower and trees were destructively harvested after only one season, therefore, this reaction was not observed.

Despite the absence of mature photosynthesizing leaves, the terminal buds on the AD shoots flushed during the same period as those on the current season's shoots of the NLD trees. This indicates maintenance of paradormancy (correlative inhibition) in the lateral buds via apical dominance exerted by the terminal bud, presumably through basipolar auxin transport (Wang et al., 1994; Faust et al., 1995; Bangerth et al., 2000). Moreover, increased levels of soil N have been shown to promote shoot growth over root growth in defoliated trees (Raitio et al., 1994). This suggests that the N concentration of the 2X treatment may have been sufficient to promote bud break in the AD trees. To prevent the young emerging leaves from developing a major demand for carbohydrates (Maggs, 1965), shoot growth was curtailed by continuous pinching and removal of the "green tips". Despite this preventative measure, a significant decline in $DM_{(shoots)}$ of the AD treatment was still observed. Choi et al. (2003) reported that the significantly lower DM allocation to the above-ground plant parts of 'Fuyu' persimmon trees

following complete defoliation in September (March in the southern hemisphere) was due to a decrease in non-structural carbohydrate (NSC) reserves. This lower DM allocation was attributed, by Larsen and Abusrewil (1983) in one-year-old graded whips of 'Delicious' apple nursery stock, to limited sorbitol and sucrose levels due to an absence of actively photosynthesizing leaves. Similar findings were reported by Nzima et al. (1999) in pistachio trees and Oliveira and Priestley (1988) in pecan trees.

Deciduous trees utilize both current photosynthates prior to leaf drop and available carbohydrate reserves during autumn/winter for maintenance respiration, root growth, stem wall thickening and bud development (Maggs, 1965; Oliveira and Priestley, 1988; Loescher et al. 1990). Thus, the significantly lower DM allocation to the shoots of the AD trees was likely due to a lack of current photosynthate supply. Furthermore, the resultant relatively low amount of stored carbohydrates was in all probability utilized for respiration during winter and not for growth, indicated by the significantly lower $FM_{(shoots)}$, supporting the findings of Palacio et al. (2014). In contrast, the NLD trees had access to both current photosynthates prior to leaf drop and stored carbohydrates due to an extended leaf drop period. Although photosynthesis and within tree C partitioning was not quantified, Van Zyl (2016) reported that photosynthesis by healthy intact leaves was maintained until 50 % leaf drop in young bearing apple trees. In support, Choi et al. (2003) found that 50 % defoliation of 'Fuyu' persimmon trees in autumn had no significant effect on the carbohydrate status of the permanent parts of the trees compared to the control (0 % defoliation). In tomato plants, Del Amor and Marcelis (2003) found that leaf photosynthesis was positively correlated with leaf Ca concentration. Since Ca translocation to the leaves of the NLD trees possibly continued until 50 % leaf drop (section 4.4), the significantly higher leaf Ca concentration of the $2X_{(NLD)}$ treatment compared to the $2X_{(AD)}$ treatment suggests that the duration of leaf photosynthesis in the present study was similar to that reported by Van Zyl (2016).

4.2. White root growth

4.2.1. Environmental effects

The relatively high white root numbers observed during autumn/winter in trees of the NLD treatments is in contrast with results reported in the northern hemisphere (Nightingale, 1935; Head, 1966; Psarras et al., 2000; Eissenstat et al., 2006), but confirms local reports (Van Zyl,

2016; Janse van Vuuren, 2018). Under northern hemisphere conditions, an absence of white root growth during autumn/winter have mainly been attributed to low soil temperatures (< 7 °C), which were not prevalent in the present study (Fig. 7). Furthermore, young absorptive roots can continue to grow independent of the time of season, provided soil temperature and moisture levels remain within an optimal range for growth (Cripps, 1970; Perry, 1971; Van Zyl, 2016). As fertigation was continuous in the present study, the observed changes in white root growth during autumn/winter were possibly under the control of factors other than soil temperature and moisture content, such as soil nutrient availability and/or endogenous tree factors.

To evaluate the effect of soil nutrient availability on apple white root growth during autumn/winter, trees were supplied with increasing levels of soil-applied $Ca(NO_3)_2$. Even though a restriction in exogenous Ca supply is known to reduce white root emergence and growth (Del Amor and Marcelis, 2003; Hawkesford et al., 2012), an increase in soil $Ca(NO_3)_2$ supply in the current study had no significant effect. This suggests that the Ca concentration of the control was sufficient to sustain growth. In contrast, a significant increase in lateral root initiation and growth was reported with increasing $CaCl_2$ treatments in cereal plants (Emanuelsson, 1984; Poovaiah and Reddy, 1991). In the present study, an increase in NO_3^--N supply could have suppressed lateral white root growth immediately after emergence, which has been demonstrated in various plant species (Zhang et al., 2007). Therefore, the non-significant white root counts between the NLD treatments could partly be ascribed to the opposing effects of Ca^{2+} and NO_3^--N in the soil solution on root growth. This also supports the hypothesis that photosynthate supply, carbohydrate storage/transport and hormonal control possibly affected root growth (Abramoff and Finzi, 2015), and confirms previous findings in various deciduous fruit crops (Cripps, 1970; Buwalda and Hutton, 1988; Glenn and Welker, 1993; Van Zyl, 2016) and forest tree stands (Hendrick and Pregitzer, 1997; Joslin et al., 2001).

4.2.2. Endogenous tree factors

White root growth was visible during much of the season in trees that experienced NLD, confirming findings of Van Zyl (2016). Two temporally unique peaks were observed in the pots. A relatively large peak was observed in late-autumn/early-winter, when white root numbers increased in March, peaked in May and gradually decreased towards the end of June, and a second, smaller peak at the end of winter (August). In contrast, Van Zyl (2016) found it

difficult to identify distinct root growth peaks in young non-bearing apple trees in the field, because the roots did not grow in close proximity to the installed minirhizotrons after the second season.

Prior to the time of defoliation, white root numbers of the AD treatment were comparable to that of the NLD treatments. As new roots rely heavily on current photosynthates (Head, 1969; Horwath et al., 1994; Lynch et al., 2013), autumn defoliation caused a drastic decline in white root numbers, in agreement with previous findings (Maggs, 1965; Head, 1969; Guo et al., 2004; Kwack et al., 2014). The development of new lateral roots is cooperatively regulated by phytohormone interactions and environmental signals; auxin being the key regulating hormone of lateral root formation (Casimiro et al., 2001; Aloni et al., 2006; Fukaki and Tasaka, 2009). Thus, the sharp contrast in white root numbers between the $2X_{(AD)}$ and NLD treatments at the beginning of winter could possibly be ascribed to a difference in current photosynthate supply and compounds actively produced in the leaves.

Trees of the NLD treatments were in full-leaf until mid-May. Prior to the onset of leaf drop, the young expanding leaves from the new growth flush were likely supplied by the more mature, actively photosynthesizing leaves (Pate, 1973; Kozlowski, 1992). Regardless of the high demand of the young shoots for carbohydrates shortly after their emergence, root growth was not negatively affected as white root numbers peaked in late-autumn/early-winter (May). Thus, the first peak in visible white roots was possibly due to a steady supply of current photosynthates (and auxins) from the actively photosynthesizing mature leaves (exporters since end of January), as well as the more recently expanded (unseasonal) leaves on the young shoots. In turn, photosynthesis and DM partitioning to the roots was possibly up-regulated through the effect of Ca in the leaves on photosynthetic capacity following uptake, and indirectly through the effect of Ca on cytokinin activity in the roots (not quantified), as previously reported by Del Amor and Marcelis (2003).

Despite the presence of actively photosynthesizing leaves, a gradual decline in white root numbers was observed in the NLD treatments until 50 % leaf drop, but numbers increased again towards the end of winter. Previous studies ascribed the decline in visible white roots to reduced root growth rates, root browning with age and/or root death and decay (Head, 1966; Atkinson, 1983; Eissenstat et al., 2000; Pregitzer et al., 2002; Guo et al., 2008; Valenzuela-Estrada et al., 2008; Comas et al., 2010; Goebel et al., 2011). Since the C utilized for

maintenance respiration over the lifetime of a root generally exceeds that used for root construction (Eissenstat and Yanai, 1997), the gradual decline in visible white roots was possibly due to the shedding of older, less efficient lower-order roots, while the eventual increase in visible white roots towards the end of winter was due to the emergence of new, more metabolically active lower-order roots (Wells and Eissenstat, 2001; Desrochers et al., 2002; Janssens et al., 2002; Eissenstat and Volder, 2005; Volder et al., 2005; Chen and Brassard, 2013). Indeed, Eissenstat et al. (2000) found that the roots of mature apple trees have a median lifespan of about 30 days, while Bouma et al. (2001) found a relatively rapid decline in the rate of nutrient (i.e. P) uptake by newly developed apple roots from 14 to 25 days after their emergence. Whether high root turnover rates carried precedence over reduced root growth rates and/or root browning with age in explaining the gradual decline in white root numbers during winter is uncertain and deserves further study.

In contrast to the NLD treatments, following a sharp decline at the beginning of winter, white root numbers of the AD treatment remained low for the rest of winter. Root DM of the $2X_{(AD)}$ treatment was significantly lower compared to the NLD treatments, while stem DM did not differ significantly between treatments. By contrast, in one-year-old 'Goldrush' kiwifruit vines, AD caused a significant decline in DM of both the above- and below-ground plant parts (Kwack et al., 2014). In agreement with our results, late-season defoliation of four-year-old 'Fuyu' persimmon trees mainly influenced DM allocation to the roots. Following 100 % defoliation in autumn, DM allocation to the roots was less than half that of the control (0 % defoliation) (Choi et al., 2003). In both these previous studies, a decrease in DM was coupled to a marked decrease in NSC reserves of the respective plant parts. Thus, the indifferent DM allocation to the stems between treatments in the present study may be explained by the additional contribution of stem internal photosynthesis (Aschan and Pfanz, 2003; Tokarz and Pilarski, 2005; Pilarski et al., 2007). In contrast, the significantly lower DM allocation to the roots of the AD treatment was possibly due to a lack of current photosynthate supply. The subsequent low amount of available carbohydrates that remained in the roots after defoliation may have been insufficient to support new growth, because the majority of the NSC pool was sequestered by incorporation into structural compounds and could not be utilized (Lacointe et al., 1993; Millard and Grelet, 2010). Carbohydrate reserves are not generally utilized for growth and/or plant metabolism during periods of active photosynthate supply (Horwath et al., 1994). Therefore, a steady supply of current photosynthates to the roots of the NLD treatments, at least until 50 % leaf drop (Van Zyl, 2016), possibly lowered the dependence of new root

growth on carbohydrate reserves. After 50 % leaf drop, the eventual increase in new white roots was possibly accomplished through the remobilization of available NSC reserves accumulated in the older, supporting roots (Guo et al., 2004; Gaudinski et al., 2009). As suggested by Lacointe et al. (1993), the most recent reserves stored closer to the vascular tissues of the roots would have been readily available.

4.3. Nutrient distribution

4.3.1. Calcium

Although an increase in soil $Ca(NO_3)_2$ supply did not have a significant impact on white root emergence, it may have had a positive influence on root longevity. McCormack et al. (2012) showed a positive correlation between fine root function and lifespan and root Ca content in temperate trees. Since white root tips are not supplied with Ca via the xylem and must absorb the Ca they require for growth directly from the soil solution (Kirkby and Pilbeam, 1984; White, 1998; White and Broadley, 2003), the Ca concentration available to the control, both from the growing medium at planting (Annexure 1, Table 1) and fertigation, may have been sufficient to support growth and improve the longevity of apple roots in the current study, as neither root Ca concentration at the end of winter nor the number of white root tips during autumn/winter differed significantly between the NLD treatments.

Although the total Ca content per tree did not differ significantly between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments, a higher % of total Ca content was allocated to the roots and reserve tissues of the stems of the $2X_{(AD)}$ treatment. Despite the absence of leaves and the relatively small number of white root tips during winter, root Ca uptake in the $2X_{(AD)}$ treatment was not negatively affected, since the Ca concentration of the roots was significantly higher compared with that of the NLD treatments. In contrast, Faust (1980) deduced that the presence of young white roots and a sufficient supply of energy from photosynthesis was required for root Ca uptake. In agreement with our results, however, Choi et al. (2003) found a significant increase in root Ca levels with an increase in defoliation percentage. Thus, under local conditions, earlier findings that a lack of current photosynthates could impair root Ca uptake through a shortage in energy supply, and indirectly, through reduced white root growth, may not be valid. It appears root Ca uptake during winter was not as dependent on a constant supply of energy from photosynthesis. In addition, substantial amounts of Ca may have been absorbed by older, brown

roots (Crider, 1933; Atkinson and Wilson, 1979, 1980; Baldi et al., 2010), especially lower-order primary roots (Wells and Eissenstat, 2003; Hishi, 2007) that occupy the greatest surface area when white root growth is sparse (Kramer and Bullock, 1966; Comerford et al., 1994).

Compared to the control and 1X treatment, an increase in soil $Ca(NO_3)_2$ supply and relatively high sap flow rates until 50 % leaf drop (section 4.4) lead to a significant increase in leaf Ca concentration in the $2X_{(NLD)}$ treatment. Since transpirational water flow directs both Ca and N translocation (Titus and Kang, 1982; Del Amor and Marcelis, 2003; White and Broadley, 2003), concurrent root NO_3^--N uptake and translocation via the xylem may have simultaneously increased the uptake and upward movement of Ca in the trees (Kirkby, 1979; Korcak, 1980; Wallace and Mueller, 1980). Moreover, Shear and Faust (1970) showed that NO_3^--N soil applications increased the translocation and subsequent accumulation of Ca^{2+} in the mature leaves of apple trees, corroborating our results.

A significantly higher DM was reported for the leaves of the $2X_{(AD)}$ treatment, but the Ca concentration of the leaves was significantly lower compared to the leaves of the $2X_{(NLD)}$ treatment. At the time of defoliation, the senescence process has not yet begun, which involves an orderly removal of nutrients from leaves destined to be shed (Titus and Kang, 1982). Therefore, the higher leaf DM of the $2X_{(AD)}$ treatment is possibly an indication that the leaves were still active up to the time they were removed, confirming previous findings (Van Zyl, 2016). The difference in leaf Ca concentration between the $2X_{(AD)}$ and $2X_{(NLD)}$ treatments was probably because the timing of soil applications coincided with different tree phenological events. Soil applications commenced after defoliation in the $2X_{(AD)}$ treatment, but before the start of leaf drop in the $2X_{(NLD)}$ treatment. Thus, while a relatively higher % of total Ca content was found in the roots and stems of the $2X_{(AD)}$ treatment, Ca translocation to the leaves of the $2X_{(NLD)}$ treatment likely continued throughout autumn/winter up to the time of leaf senescence. Since these trees experienced a rather extended leaf drop period, and Ca is not readily redistributed from the leaves to the permanent parts of the trees after its delivery there (Biddulph et al., 1961; Mengel, 2002; White and Broadley, 2003; Gilliham et al., 2011), it is likely that a significant amount of absorbed Ca was lost at leaf drop, confirming previous findings (Terblanche, 1972; Conradie, 1981; Stassen and Stadler, 1988; Kangueehi et al., 2011). Based on the assumption that very little (< 5 %) redistribution of Ca from the leaves to the permanent parts of the trees occur (Hanekom, 1973; Peuke, 2010), approximately 37 % of the total uptake of Ca was lost through NLD, whereas 31 % of the total uptake of Ca was lost

when the leaves were removed before NLD commenced. The % of total Ca content lost in the $2X_{(NLD)}$ treatment at the end of August is considerably higher than the 29 % reported by Terblanche (1972). However, in his study, 100 % leaf drop was already reached at the beginning of August and the trees did not receive additional soil Ca applications in autumn. Locally, it therefore appears that an extended leaf drop period following soil $Ca(NO_3)_2$ applications could have a negative impact on Ca reserve accumulation in the roots and stems of young non-bearing apple trees during autumn/winter. This was also demonstrated by the significantly higher % of total Ca content allocated to the reserve tissues of the $2X_{(AD)}$ treatment.

The major difference in Ca reserve content between the $2X_{(AD)}$ and $2X_{(NLD)}$ treatments resided in the permanent structural components of the trees. The % of total Ca content in the stems of the $2X_{(AD)}$ treatment was significantly higher at 38 % (or 55 % excluding the leaves) compared to the 29 % (or 47 % excluding the leaves) in the stems of the $2X_{(NLD)}$ treatment, while the difference in % of total Ca content in the roots between these two treatments (20 % or 32 % excluding the leaves in the $2X_{(NLD)}$ treatment, and 25 % or 36 % excluding the leaves in the $2X_{(AD)}$ treatment), although significant, only amounted to ± 4 %. Even with additional soil Ca applications, the 47 % contribution of the stems in the $2X_{(NLD)}$ treatment fell well below the 58 % reported by Terblanche (1972), while the 32 % contribution of the roots compared well with the results of Terblanche (1972) at 31 %. Since the % of total Ca content in the shoots of the $2X_{(AD)}$ treatment (6 % or 9 % excluding the leaves) was significantly lower compared with that of the $2X_{(NLD)}$ treatment (13 % or 21 % excluding the leaves), Ca reserve accumulation in the shoots did not seem to benefit from early leaf drop. As the % of total Ca content in the reserve tissues of the $2X_{(AD)}$ treatment was ± 7 % higher compared with that of the $2X_{(NLD)}$ treatment, there seems to be differences in the pattern of Ca reserve accumulation in relation to the duration of leaf drop under local conditions.

4.3.2. Nitrogen

In various deciduous fruit crops (Kotzé and De Villiers, 1989; Conradie, 1990; Cheng et al., 2001; El-Jendoubi et al., 2013), including apple (Terblanche, 1972; Neilsen et al., 2001; Kangueehi et al., 2011), N remobilization from senescing leaves to the reserve tissues prior to leaf abscission during autumn is typical. The duration and extent of this occurrence, however, has been shown to depend on the timing and amount of soil N supply. In one-year-old, potted,

M26 apple rootstocks, Millard and Thomson (1989) found that plants that received a sufficient supply of N from the soil throughout summer but none in autumn, displayed a typical pattern of leaf senescence and N remobilization from the leaves to the reserve tissues for storage over winter. In contrast, in plants that received an additional supply of N in autumn, delayed leaf senescence as well as reduced rates of N remobilization from the leaves were apparent. In the present study, although an extended leaf drop period was observed, no differences in leaf drop duration were observed between the control and NLD treatments. However, leaf N concentration of the 1X and $2X_{(NLD)}$ treatments were significantly higher compared to the control. Since the N concentrations of the roots, stems and shoots of the 1X and $2X_{(NLD)}$ treatments were significantly higher compared to the control, a considerable amount of absorbed N was probably still remobilized from the leaves for storage prior to leaf abscission. In support, although the total N content (g DM) per tree was significantly higher in the $2X_{(NLD)}$ treatment compared to the $2X_{(AD)}$ treatment, the % of total N content in the leaves of the $2X_{(NLD)}$ treatment was significantly lower compared to that of the $2X_{(AD)}$ treatment (11 % vs 24 %), while the % of total N content in the shoots (11 % vs 4 %) and roots (42 % vs 35 %) were significantly higher in the $2X_{(NLD)}$ treatment compared to the $2X_{(AD)}$ treatment.

Furthermore, as ribulose 1,5 bisphosphate carboxylase/oxygenase (RUBISCO) accounting for the majority of soluble leaf protein (Titus and Kang, 1982; Millard and Thomson, 1989), plays such a pivotal role in photosynthetic carbon dioxide (CO_2) assimilation in the leaves (Taiz et al., 2015), the extended leaf drop period and the significant higher leaf N concentrations of the 1X and $2X_{(NLD)}$ treatments compared to the control, possibly enabled a substantial increase in carbohydrate reserve accumulation via leaf photosynthesis prior to leaf drop. During periods of low energy demand, excess photosynthates are stored as reserves, and these reserves in turn, are utilized for energy when demands exceed supply (Richardson et al., 2013). Therefore, the linear increase in root N concentration and the concurrent linear decrease in root DM among the NLD treatments during winter, was likely coupled to the utilization of stored carbohydrates in the roots for N assimilation when current supplies were lacking. Compared to the $2X_{(NLD)}$ treatment, early leaf removal prior to the onset of NLD caused a significant decline in root N concentration and root FM and DM, also reported by Guo et al. (2004). Since a decrease in root DM is indicative of a decrease in carbohydrate reserve concentration (Choi et al., 2003), the significantly lower root N concentration as well as % of total N content in the roots of the $2X_{(AD)}$ treatment was possibly the result of both reduced uptake due the effects of AD on root

growth during winter and lower available amounts of stored carbohydrates in the roots for N assimilation due to early leaf loss.

4.4. Calcium uptake and distribution in relation to sap flow dynamics

Although sap flow was not directly measured in the present study, a direct link between diurnal stem diameter fluctuations (i.e. shrinkage and swelling) and sap flow dynamics exist as a result of the daily course of transpiration in trees (Huguet et al., 1992; Zweifel et al., 2000, 2001; Steppe and Lemeur, 2004; Steppe et al., 2005; De Swaef et al., 2009; De Swaef and Steppe, 2010). When water reserves from the tissues surrounding the xylem in the stem become depleted due to a decrease in xylem water potential caused by relatively high rates of transpiration during the day, the stem shrinks, reaching a minimum diameter around mid-afternoon. As the stem swells due to replenishment of the storage tissues caused by relatively high root water uptake rates during the night when transpiration rates are generally low, it reaches a maximum diameter early in the morning (Huguet et al., 1992; Zweifel et al., 2000, 2001).

The trend in MDS between the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments were similar from the time of defoliation (end of April). This gave an indication, albeit indirectly, that sap flow dynamics was not significantly affected by early leaf removal. However, the MDS of the $2X_{(AD)}$ treatment was lower compared to the $2X_{(NLD)}$ treatment from the time of defoliation until 50 % leaf drop (July). Thereafter, the difference in MDS between treatments became negligible towards the end of winter. Unfortunately leaf transpiration was not directly measured, but Huguet et al. (1992) showed that low transpiration rates of stressed apple trees resulted in very small MDS values compared to trees that were left unstressed. Based on this information, it is proposed that leaf transpiration until 50 % leaf drop was adequate to maintain relatively high sap flow rates in the $2X_{(NLD)}$ treatment. As leaf drop progressed, transpiration decreased to such an extent that sap flow rates between treatments became comparable.

Since a difference in MDS between treatments only occurred before 50 % leaf drop, it is likely that the majority of Ca was translocated to the leaves of the $2X_{(NLD)}$ treatment during this period. The duration of Ca influx to the leaves is, however, uncertain. A significant decline in Ca influx after leaf maturity was previously reported, even though the leaves were still actively transpiring (Mengel and Kirkby, 1987). A relatively high concentration of $Ca(NO_3)_2$ was

applied to the soil within three weeks of defoliation of the AD trees. Despite the absence of leaves, a significant increase in root Ca concentration was found, confirming findings of Choi et al. (2003). Since the AD trees were completely leafless when $Ca(NO_3)_2$ was applied, leaf transpiration-driven sap flow could not have been responsible for root Ca uptake from the soil. However, since the MDS of the trees remained above zero value, active sap flow in the xylem was probably still taking place, albeit at a lower intensity, should an equilibrium in water potential between the xylem and phloem of the AD trees have been maintained (Sevanto et al., 2003). This was, however, not quantified.

Stem transpiration via natural openings (mainly open lenticels and stomata, but also bud scales and leaf scars) in the outer bark layers of trees can contribute significantly to transpiration, but its contribution is generally inferior to leaf transpiration (Groh et al., 2002; Pfanz et al., 2002). Although the trees experienced relatively high daytime temperatures, a significant contribution of stem transpiration to upward xylem sap flow during winter is doubtful, as stem lenticels are mostly closed by an intact, suberized closing layer during dormancy (Rosner and Kartusch, 2003). This was, however, not quantified. Under conditions of little or no transpiration, positive root pressure can contribute to upward xylem sap flow in well-watered trees (Wegner, 2014) and are capable of delivering nutrients (e.g. K, Ca) from the roots to the leaves and fruit of various crops, especially at night (Palzkill and Tibbitts, 1977; Guttridge et al. 1981; Tanner and Beevers, 2001; Lee et al., 2004). Under these conditions, the transpiration stream is too weak to remove excess ions from the xylem and stellar apoplast. A resultant concentration gradient favours diffusion back into the cortical apoplast. The backflow of ions from the stellar apoplast to the cortical apoplast is prevented by endodermal Casparian bands, causing them to accumulate in the xylem. As a consequence, root pressure builds via energy-dependent osmotic water flow across the plasma membranes of xylem parenchyma cells into the xylem causing or contributing to upwards xylem sap flow (De Boer and Volkov, 2003; Enstone et al., 2003; Wegner, 2014; Singh, 2016). Concurrent with the present experiment, young white roots were sampled at bi-weekly to monthly intervals from the trees of the control and $2X_{(NLD)}$ treatment to quantify Ca uptake and distribution in apple root tips during winter by scanning electron microscopy and wavelength-dispersive x-ray spectroscopy (Paper 2). Results showed that the Ca concentration in the xylem was significantly lower compared to the cortex in roots harvested after 50 % leaf drop. Moreover, the differences in Ca concentration in the xylem between roots harvested before and after 50 % leaf drop were not statistically significant. Based on these findings and those of others in tomato (Ho, 1989), maize (Van de Geijn and Smeulders, 1981)

and apple (Tromp and Van Vuure, 1993), it seems unlikely that root pressure alone could contribute significantly to upward xylem sap flow and Ca translocation in trees of the $2X_{(AD)}$ treatment and $2X_{(NLD)}$ treatment after 50 % leaf drop. However, as root pressure was not recorded, this remains uncertain.

Between the onset and 50 % leaf drop, the relatively high rates of leaf transpiration-driven sap flow possibly increased the rate of root Ca uptake and translocation via the apoplast (White, 2001; White and Broadley, 2003; Gilliham et al., 2011), permitting the leaves and shoots (unseasonal) to accumulate substantial amounts of Ca. The possible formation of uncharged or negatively charged Ca complexes in the roots could also have contributed to high rates of Ca translocation to the leaves (Bradfield, 1976). Since the roots are relatively active during autumn/winter under local conditions (Terblanche et al., 1979; Van Zyl, 2016), the utilization of Ca in proportion to their growth and/or metabolic demand could have created temporary open exchange sites in the root apoplast and xylem conduits (Biddulph et al., 1961), steepening the gradient between the apoplast/xylem vessels and the surrounding cells. This possibly allowed the relatively low rates of xylem sap flow in the $2X_{(AD)}$ treatment and $2X_{(NLD)}$ treatment after 50 % leaf drop to drive root Ca uptake from the soil. Since the % of total Ca content allocated to the roots and stems of the $2X_{(AD)}$ treatment was significantly higher compared to the $2X_{(NLD)}$ treatment, it is speculated that the continuous deposition of Ca in the roots of the $2X_{(AD)}$ treatment eventually saturated the ion exchange sites within the cell walls of the root apoplast and xylem conduits (Biddulph, 1967; Haynes, 1980; Sattelmacher, 2001), thereby allowing the relatively low rate of sap flow in the xylem to deliver excess Ca^{2+} ions, and possibly also Ca-citrate/malate complexes (Prima-Putra and Botton, 1998), to the wood and bark tissues of the stems for storage.

5. Conclusion

An increase in soil $Ca(NO_3)_2$ supply in autumn did not have a significant effect on white root growth in young, potted, non-bearing apple trees during winter, but the presence of leaves did. Early leaf loss (via defoliation in April) caused a drastic decline in white root numbers during winter. This shows that an abundance of functional leaves in young apple trees in autumn prior to leaf drop, is essential for the maintenance and growth of white roots during winter under local conditions, in agreement with Wilcox (1968).

Unexpectedly, low white root numbers during winter did not have a negative impact on Ca uptake and reserve accumulation in defoliated trees. This may be an indication that substantial Ca uptake by older, brown roots occurred during this period. The % of total Ca content in the reserve tissues of the $2X_{(AD)}$ treatment was significantly higher at the end of winter compared to the $2X_{(NLD)}$ treatment. As the latter trees experienced an extended leaf drop period, a substantial fraction of the total Ca content in the trees was lost via transport to the leaves at the expense of Ca distribution to the reserve tissues during winter. The high % of total Ca content in the leaves was possibly the result of relative high rates of leaf transpiration-driven sap flow prior to 50 % leaf drop, consequently lowering the amount of available Ca in the soil for reserve accumulation during the latter part of winter.

Despite the absence of transpiring leaves in the $2X_{(AD)}$ trees, substantial root Ca uptake and reserve accumulation were evident. This suggests that leaf transpiration-driven sap flow is not essential for root Ca uptake and translocation during winter dormancy. The mechanism responsible for sustained sap flow in leafless apple trees during winter could, however, not be established. Future studies should include quantification of root pressure. The simultaneous use of sap flow sensors (e.g. heat balance sensors) and instruments that measure continuous stem diameter variations (e.g. point dendrometers or linear variable displacement transducers) offers an opportunity to indirectly measure root pressure non-destructively, and at the same time, directly measure sap flow dynamics in the xylem (De Swaef and Steppe, 2012; De Swaef et al., 2013). Physiological measurements i.e. photosynthesis and transpiration (both leaf and stem) is also advised. Since all trees were destructively harvested at the end of winter, the effect of the differential patterns of Ca reserve accumulation in the $2X_{(AD)}$ and $2X_{(NLD)}$ treatments on the Ca concentration of the new growth (leaves, shoots and fruit) in young apple trees in the following season could not be established and deserves further attention.

Acknowledgements

Yara Africa Fertilizer (Pty). Ltd. for their support and funding of this experiment. Dr. N. Taylor (University of Pretoria, SA) and Dr. S. Dzikiti (CSIR, Stellenbosch, SA) for their assistance with the dendrometer study.

References

Abramoff, R.Z., Finzi, A.C., 2015. Are above- and below-ground phenology in sync? New Phytol. 205, 1054–1061.

Aloni, R., Aloni, E., Langhans, M., Ullrich, C.I., 2006. Role of cytokinin and auxin in shaping root architecture: Regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann. Bot. 97, 883–893.

Anderson, L.J., Comas, L.H., Lakso, A.N., Eissenstat, D.M., 2003. Multiple risk factors in root survivorship: A 4-year study in Concord grape. New Phytol. 158, 489–501.

Aschan, G., Pfanz, H., 2003. Non-foliar photosynthesis – a strategy of additional carbon acquisition. Flora 198, 81–97.

Atkinson, C.J., Ruiz, L.P., Mansfield, T.A., 1992. Calcium in xylem sap and the regulation of its delivery to the shoot. J. Exp. Bot. 43, 1315–1324.

Atkinson, D., 1972. Seasonal periodicity of black current root growth and the influence of simulated mechanical harvesting. J. Hort. Sci. 47, 165–172.

Atkinson, D., 1983. The growth, activity and distribution of the fruit tree root system. Plant Soil 71, 23–35.

Atkinson, D., Wilson, S.A., 1979. The root-soil interface and its significance for fruit tree roots of different ages, in: Harley, J.L., Russel, R.S. (Eds.), The Soil-Root Interface. Academic Press, London, pp. 259–271.

Atkinson, D., Wilson, S.A., 1980. The growth and distribution of fruit tree roots: Some consequences for nutrient uptake, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 137–150.

Baldi, E., Wells, C.E., Marangoni, B., 2010. Nitrogen absorption and respiration in white and brown peach roots. J. Plant Nutr. 33, 461–469.

Bangerth, F., Li, C., Gruber, J., 2000. Mutual interaction of auxin and cytokinins in regulating correlative dominance. Plant Growth Regul. 32, 205–217.

Beikircher, B., Mayr, S., 2013. Winter peridermal conductance of apple trees: Lammas shoots and spring shoots compared. Trees 27, 707–715.

Bell, C.W., Biddulph, O., 1963. Translocation of calcium. Exchange versus mass flow. Plant

Physiol. 38, 610–614.

Biddulph, O., Nakayama, F.S., Cory, R., 1961. Transpiration stream and ascension of calcium. Plant Physiol. 36, 429–436.

Biddulph, S.F., 1967. A microautoradiographic study of ^{45}Ca and ^{35}S distribution in the intact bean root. Planta 74, 350–367.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Bradfield, E.G., 1976. Calcium complexes in the xylem sap of apple shoots. Plant Soil 44, 495–499.

Buwalda, J.G., Hutton, R.C., 1988. Seasonal changes in root growth of kiwifruit. Sci. Hort. 36, 251–260.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inzé, D., Sandberg, G., Casero, P.J., Bennet, M., 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell 13, 843–852.

Chen, H.Y.H., Brassard, B.W., 2013. Intrinsic and extrinsic controls of fine root life span. Crit. Rev. Plant Sci. 32, 151–161.

Cheng, L., Dong, S., Guak, S., Fuchigami, L.H., 2001. Effects of nitrogen fertigation on reserve nitrogen and carbohydrate status and regrowth performance of pear nursery plants. Acta Hort. 564, 51–62.

Cheng, L., Fuchigami, L.H., 2002. Growth of young apple trees in relation to reserve nitrogen and carbohydrates. Tree Physiol. 22, 1297–1303.

Cheng, L., Raba, R., 2009. Accumulation of macro- and micronutrients and nitrogen demand-supply relationship of 'Gala'/'Malling 26' apple trees grown in sand culture. J. Am. Soc. Hort. Sci. 134, 3–13.

Choi, S.T., Park, D.S., Song, W.D., Kang, S.M., Shon, G.M., 2003. Effect of different degrees of defoliation on fruit growth and reserve accumulation in young 'Fuyu' trees. Acta Hort. 99–104.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

Codesido, V., Fernandez-Lopez, J., 2009. Genetic variation in seasonal growth patterns in radiata pine in Galicia (Northern Spain). For. Ecol. Manage. 257, 518–526.

Comas, L.H., Bauerle, T.L., Eissenstat, D.M., 2010. Biological and environmental factors controlling root dynamics and function: effects of root ageing and soil moisture. Aust. J. Grape Wine Res. 16, 131–137.

Comerford, N.B., Smethurst, P.J., Escamilla, J.A., 1994. Nutrient uptake by woody root systems. New Zeal. J. For. Sci. 24, 195–212.

Conradie, W.J., 1981. Seasonal uptake of nutrients by Chenin blanc in sand culture: II. Phosphorus, potassium, calcium and magnesium. South African J. Enol. Vitic. 2, 7–13.

Conradie, W.J., 1990. Distribution and translocation of nitrogen absorbed during late spring by two-year-old grapevines grown in sand culture. Am. J. Enol. Vitic. 41, 241–250.

Contador, M.L., Comas, L.H., Metcalf, S.G., Stewart, W.L., Gomez, I.P., Negron, C., Lampinen, B.D., 2015. Root growth dynamics linked to above-ground growth in walnut (*Juglans regia*). Ann. Bot. 116, 49–60.

Côté, B., Hendershot, W.H., Fyles, J.W., Roy, A.G., Bradley, R., Biron, P.M., Courchesne, F., 1998. The phenology of fine root growth in maple-dominated ecosystem: Relationships with some soil properties. Plant Soil 201, 59–69.

Crider, F.J., 1933. Selective absorption of ions not confined to young rootlets. Science 78, 169.

Cripps, J.E.L., 1970. A seasonal pattern of apple root growth in western Australia. J. Hort. Sci. 45, 153–161.

De Boer, A.H., Volkov, V., 2003. Logistics of water and salt transport through the plant: structure and functioning of the xylem. Plant Cell Environ. 26, 87–101.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hort. Rev. 40, 107–146.

De Swaef, T., Hanssens, J., Cornelis, A., Steppe, K., 2013. Non-destructive estimation of root pressure using sap flow, stem diameter measurements and mechanistic modelling. Ann. Bot. 111, 271–282.

De Swaef, T., Steppe, K., 2010. Linking stem diameter variations to sap flow, turgor and water

potential in tomato. Funct. Plant Biol. 37, 429–438.

De Swaef, T., Steppe, K., 2012. Sap flow, stem diameter variations and mechanistic modelling have potential to assess root pressure. Acta Hort. 951, 111–116.

De Swaef, T., Steppe, K., Lemeur, R., 2009. Determining reference values for stem water potential and maximum daily trunk shrinkage in young apple trees based on plant responses to water deficit. Agric. Water Manag. 96, 541–550.

Del Amor, F.M., Marcelis, L.F.M., 2003. Regulation of nutrient uptake, water uptake and growth under calcium starvation and recovery. J. Hort. Sci. Biotechnol. 78, 343–349.

Del Amor, F.M., Marcelis, L.F.M., 2006. Differential effect of transpiration and Ca supply on growth and Ca concentration of tomato plants. Sci. Hort. 111, 17–23.

Desrochers, A., Landhäusser, S.M., Lieffers, V.J., 2002. Coarse and fine root respiration in aspen (*Populus tremuloides*). Tree Physiol. 22, 725–732.

Eissenstat, D.M., Bauerle, T.L., Comas, L.H., Neilsen, D., Neilsen, G.H., Lakso, A.N., Smart, D.R., 2006. Seasonal patterns of root growth in relation to shoot phenology in grape and apple. Acta Hort. 721, 21–26.

Eissenstat, D.M., Volder, A., 2005. The efficiency of nutrient acquisition over the life of a root, in: Bassirirad, H. (Ed.), Nutrient Acquisition by Plants. Springer, Berlin Heidelberg, pp. 185–220.

Eissenstat, D.M., Wells, C.E., Wang, L., 2001. Root efficiency and mineral nutrition in apple. Acta Hort. 165–183.

Eissenstat, D.M., Wells, C.E., Yanai, R.D., Whitbeck, J.L., 2000. Building roots in a changing environment: Implications for root longevity. New Phytol. 147, 33–42.

Eissenstat, D.M., Yanai, R.D., 1997. Ecology of root life span. Adv. Ecol. Res. 27, 1–60.

El-Jendoubi, H., Abadía, J., Abadía, A., 2013. Assessment of nutrient removal in bearing peach trees (*Prunus persica* L. Batsch) based on whole tree analysis. Plant Soil 369, 421–437.

Emanuelsson, J., 1984. Root growth and calcium uptake in relation to calcium concentration. Plant Soil 78, 325–334.

Engels, C., 1999. Regulation of xylem transport of calcium from roots to shoot of maize by growth-related demand. J. Plant Nutr. Soil Sci. 162, 287–294.

Enstone, D.E., Peterson, C.A., Ma, F., 2003. Root endodermis and exodermis: Structure, function, and responses to the environment. J. Plant Growth Regul. 21, 335–351.

Faust, M., 1980. Interaction between nutrient uptake and photosynthesis, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 193–199.

Faust, M., Liu, D., Wang, S.Y., Stutte, G.W., 1995. Involvement of apical dominance in winter dormancy of apple buds. Acta Hort. 395, 47–56.

Ferguson, I.B., 1980. The uptake and transport of calcium in the fruit tree, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 183–192.

Ferguson, I.B., Bollard, E.G., 1976. The movement of calcium in woody stems. Ann. Bot. 40, 1057–1065.

Ferguson, I.B., Turner, N.A., 1981. Mobilization of macro-nutrients in cuttings of kiwifruit (*Actinidia chinensis* Planch.). Ann. Bot. 47, 229–237.

Fukaki, H., Tasaka, M., 2009. Hormone interactions during lateral root formation. Plant Mol. Biol. 69, 437–449.

Gaudinski, J.B., Torn, M.S., Riley, W.J., Swanston, C., Trumbore, S.E., Joslin, J.D., Majdi, H., Dawson, T.E., Hanson, P.J., 2009. Use of stored carbon reserves in growth of temperate tree roots and leaf buds: Analyses using radiocarbon measurements and modeling. Glob. Chang. Biol. 15, 992–1014.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Glenn, D.M., Welker, W.V., 1993. Root development patterns in field grown peach trees. J. Am. Soc. Hort. Sci. 118, 362–365.

Goebel, M., Hobbie, S., Bulaj, B., Zadworny, M., Archibald, D., Oleksyn, J., Reich, P., Eissenstat, D.M., 2011. Decomposition of the finest root branching orders: Linking carbon and nutrient dynamics belowground to fine root function and structure. Ecol. Monogr. 81, 89–102.

Groh, B., Hübner, C., Lendzian, K.J., 2002. Water and oxygen permeance of phellems isolated

from trees: The role of waxes and lenticels. Planta 215, 794–801.

Gu, J., Wei, X., Wang, J., Dong, X., Wang, Z., 2015. Marked differences in standing biomass, length density, anatomy and physiological activity between white and brown roots in *Fraxinus mandshurica* Rupr. plantation. Plant Soil 392, 267–277.

Guak, S., Neilsen, D., Millard, P., Wendler, R., Neilsen, G.H., 2003. Determining the role of N remobilization for growth of apple (*Malus domestica* Borkh.) trees by measuring xylem-sap N flux. J. Exp. Bot. 54, 2121–2131.

Guo, D., Mitchell, R.J., Withington, J.M., Fan, P.-P., Hendricks, J.J., 2008. Endogenous and exogenous controls of root life span, mortality and nitrogen flux in a longleaf pine forest: root branch order predominates. J. Ecol. 96, 737–745.

Guo, D.L., Mitchell, R.J., Hendricks, J.J., 2004. Fine root branch orders respond differentially to carbon source-sink manipulations in a longleaf pine forest. Oecologia 140, 450–457.

Guttridge, C.G., Bradfield, E.G., Holder, R., 1981. Dependence of calcium transport into strawberry leaves on positive pressure in the xylem. Ann. Bot. 48, 473–480.

Hanekom, A.N., 1973. Opname van kalsium-45 deur appelbome by verskillende vogpeile en die induksie van biterpit. Ph.D. dissertation, Faculty of Natural Science, Randse Afrikaanse Universiteit.

Hanger, B.C., 1979. The movement of calcium in plants. Commun. Soil Sci. Plant Anal. 10, 171–193.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Moller, I.S., White, P., 2012. Functions of macronutrients, Marschner's Mineral Nutrition of Higher Plants. doi:10.1016/B978-0-12-384905-2.00006-6.

Haynes, R.J., 1980. Ion exchange properties of roots and ionic interactions within the root apoplasm: Their role in ion accumulation by plants. Bot. Rev. 46, 75–99.

Head, G.C., 1966. Estimating seasonal changes in the quantity of white suberized root on fruit trees. J. Hort. Sci. 41, 197–206.

Head, G.C., 1967. Effects of seasonal changes in shoot growth on the amount of unsuberized root on apple and plum trees. J. Hort. Sci. 42, 169–180.

Head, G.C., 1969. The effects of fruiting and defoliation on seasonal trends in new root production on apple trees. J. Hort. Sci. 44, 175–181.

Heide, O.M., Prestrud, A.K., 2005. Low temperature, not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol. 25, 109–114.

Hendrick, R.L., Pregitzer, K.S., 1997. The relationship between fine root demography and the soil environment in northern hardwood forests. Ecoscience 4, 99–105.

Hishi, T., 2007. Heterogeneity of individual roots within the fine root architecture: Causal links between physiological and ecosystem functions. J. For. Res. 12, 126–133.

Ho, L.C., 1989. Environmental effects on the diurnal accumulation of ^{45}Ca by young fruit and leaves of tomato plants. Ann. Bot. 63, 281–288.

Horwath, W.R., Pregitzer, K.S., Paul, E.A., 1994. ^{14}C Allocation in tree-soil systems. Tree Physiol. 14, 1163–1176.

Huguet, J.G., Li, S.H., Lorendeau, J.Y., Pelloux, G., 1992. Specific micromorphometric reactions of fruit trees to water stress and irrigation scheduling automation. J. Hort. Sci. 67, 631–640.

Janse van Vuuren, L., 2018. Quantifying the effect of commercial nitrogen formulations BlackUreaTM/BlackDAPTM as alternative nitrogen source on fruit tree physiology, yield and fruit quality of two apple cultivars. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Janssens, I.A., Sampson, D.A., Curiel-Yuste, J., Carrara, A., Ceulemans, R., 2002. The carbon cost of fine root turnover in a Scots pine forest. For. Ecol. Manage. 168, 231–240.

Joslin, J.D., Wolfe, M.H., Hanson, P.J., 2001. Factors controlling the timing of root elongation intensity in a mature upland oak stand. Plant Soil 228, 201–212.

Kandiah, S., 1979. Turnover of carbohydrates in relation to growth in apple trees. II. Distribution of ^{14}C assimilates labelled in autumn, spring and summer. Ann. Bot. 44, 185–195.

Kangueehi, G.N., 2008. Nutrient requirement and distribution of intensively grown 'Brookfield Gala' apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Kangueehi, G.N., Stassen, P.J.C., Theron, K.I., Wooldridge, J., 2011. Macro and micro element requirements of young and bearing apple trees under drip fertigation. South African J. Plant Soil 28, 136–141.

Kirkby, E.A., 1979. Maximizing calcium uptake by plants. Commun. Soil Sci. Plant Anal. 10, 89–113.

Kirkby, E.A., Pilbeam, D.J., 1984. Calcium as a plant nutrient. Plant Cell Environ. 7, 397–405.

Korcak, R., 1980. The importance of calcium and nitrogen source in fruit tree nutrition, in: Atkinson, D., Jackson, J.E., Sharples, R.O. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 267–277.

Kotzé, W.A.G., De Villiers, J., 1989. Seasonal uptake and distribution of nutrient elements by kiwifruit vines 1. Macronutrients. South African J. Plant Soil 6, 256–264.

Kozlowski, T.T., 1964. Shoot growth in woody plants. Bot. Rev. 30, 335–392.

Kozlowski, T.T., 1992. Carbohydrate sources and sinks in woody plants. Bot. Rev. 58, 107–222.

Kramer, P.J., Bullock, H.C., 1966. Seasonal variations in the proportions of suberized and unsuberized roots of trees in relation to the absorption of water. Am. J. Bot. 53, 200–204.

Kwack, Y., Kim, H.L., Chae, W., Kim, S.H., Lee, Y., Kim, J.G., 2014. Carbohydrate reserves of non-fruiting young kiwifruit vines as affected by early artificial defoliation. Hort. Environ. Biotechnol. 55, 462–470.

Lacointe, A., Kajji, A., Daudet, F., Archer, P., Frossard, J., Saint-Joanis, B., Vandame, M., 1993. Mobilization of carbon reserves in young walnut trees. Acta Bot. Gall. 140, 435–441.

Larsen, F.E., Abusrewil, G.S., 1983. Pre- and post-storage levels of sorbitol and sugars in 'Delicious' apple nursery stock as affected by chemical- or hand-defoliation. Sci. Hort. 20, 355–360.

Lee, S.H., Singh, A.P., Chung, G.C., Ahn, S.J., Noh, E.K., Steudle, E., 2004. Exposure of roots of cucumber (*Cucumis sativus*) to low temperature severely reduces root pressure, hydraulic conductivity and active transport of nutrients. Physiol. Plant. 120, 413–420.

Link, S.O., Thiede, M.E., Van Bavel, M.G., 1998. An improved strain-gauge device for continuous field measurement of stem and fruit diameter. J. Exp. Bot. 49, 1583–1587.

Loescher, W.H., Mccamant, T., Keller, J.D., 1990. Carbohydrate reserves, translocation, and storage in woody plant roots. HortScience 25, 274–281.

Lynch, D.J., Matamala, R., Iversen, C.M., Norby, R.J., Gonzalez-Meler, M.A., 2013. Stored

carbon partly fuels fine-root respiration but is not used for production of new fine roots. New Phytol. 199, 420–430.

Maggs, D.H., 1965. Growth rates in relation to assimilate supply and demand: II. The effect of particular leaves and growing regions in determining the dry matter distribution in young apple trees. J. Exp. Bot. 16, 387–404.

McCormack, M.L., Adams, T.S., Smithwick, E.A.H., Eissenstat, D.M., 2012. Predicting fine root lifespan from plant functional traits in temperate trees. New Phytol. 195, 823–831.

McLaughlin, S.B., Wimmer, R., 1999. Calcium physiology and terrestrial ecosystem processes. New Phytol. 142, 373–417.

Mengel, K., 2002. Alternative or complementary role of foliar supply in mineral nutrition. Acta Hort. 594, 33–47.

Mengel, K., Kirkby, E.A., 1987. Principles of plant nutrition, 4th ed. International Potash Institute, Switzerland.

Midgley, S.J.E., Lötze, E., 2011. Climate change in the Western Cape of South Africa: Trends, projections and implications for chill unit accumulation. Acta Hort. 903, 1127–1134.

Millard, P., Grelet, G., 2010. Nitrogen storage and remobilization by trees: Ecophysiological relevance in a changing world. Tree Physiol. 30, 1083–1095.

Millard, P., Thomson, C.M., 1989. The effect of the autumn senescence of leaves on the internal cycling of nitrogen for the spring growth of apple trees. J. Exp. Bot. 40, 1285–1289.

Montagnoli, A., Di Iorio, A., Terzaghi, M., Trupiano, D., Scippa, G.S., Chiantante, D., 2014. Influence of soil temperature and water content on fine-root seasonal growth of European beech natural forest in Southern Alps, Italy. Eur. J. For. Res. 133, 957–968.

Montanaro, G., Dichio, B., Xiloyannis, C., 2010. Significance of fruit transpiration on calcium nutrition in developing apricot fruit. J. Plant Nutr. Soil Sci. 173, 618–622.

Neilsen, D., Millard, P., Neilsen, G.H., Hogue, E.J., 2001. Nitrogen uptake, efficiency of use, and partitioning for growth in young apple trees. J. Am. Soc. Hort. Sci. 126, 144–150.

Nightingale, G.T., 1935. Effects of temperature on growth, anatomy, and metabolism of apple and peach roots. Bot. Gaz. 96, 581–639.

Nzima, M.D.S., Martin, G.C., Nishijima, C., 1999. Effect of fall defoliation and spring shading

on shoot carbohydrate and growth parameters among individual branches of alternate bearing 'Kerman' pistachio trees. J. Amer. Soc. Hort. Sci. 124, 52–60.

Oliveira, C.M., Priestley, C.A., 1988. Carbohydrate reserves in deciduous fruit trees. Hort. Rev. 10, 403–430.

Palacio, S., Hoch, G., Sala, A., Korner, C., Millard, P., 2014. Does carbon storage limit tree growth? New Phytol. 201, 1096–1100.

Palzkill, D.A., Tibbitts, T.W., 1977. Evidence that root pressure flow is required for calcium transport to head leaves of cabbage. Plant Physiol. 60, 854–856.

Pate, J.S., 1973. Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol. Biochem. 5, 109–119.

Perry, T.O., 1971. Dormancy of trees in winter. Science 171, 29–36.

Peuke, A.D., 2010. Correlations in concentrations, xylem and phloem flows, and partitioning of elements and ions in intact plants. A summary and statistical re-evaluation of modelling experiments in *Ricinus communis*. J. Exp. Bot. 61, 635–655.

Pfanz, H., Aschan, G., Langenfeld-Heyser, R., Wittmann, C., Loose, M., 2002. Ecology and ecophysiology of tree stems: Corticular and wood photosynthesis. Naturwissenschaften 89, 147–162.

Pilarski, J., Tokarz, K., Kocurek, M., 2007. Comparison of photosynthetic pigment contents in stems and leaves of fruit trees: cherry, sweet cherry, common plum, and walnut tree. Folia Hort. 19, 53–65.

Poovaiah, B.W., Reddy, A.N., 1991. Calcium and root development: Importance of calcium in signal transduction, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 205–227.

Pregitzer, K.S., Deforest, J.L., Burton, A.J., Allen, M.F., Ruess, W.R., Hendrick, R.L., 2002. Fine root architecture of nine North American trees. Ecol. Monogr. 72, 293–309.

Prima-Putra, D., Botton, B., 1998. Organic and inorganic compounds of xylem exudates from five woody plants at the stage of bud breaking. J. Plant Physiol. 153, 670–676.

Psarras, G., Merwin, I.A., Lakso, A.N., Ray, J.A., 2000. Root growth phenology, root longevity, and rhizosphere respiration of field grown 'Mutsu' apple trees on 'Malling 9' rootstock. J. Am. Soc. Hort. Sci. 125, 596–602.

Raese, J.T., Staiff, D.C., 1990. Fruit calcium, quality and disorders of apples (*Malus domestica*) and pears (*Pyrus communis*) influenced by fertilizers, in: Van Beusichem, M.L. (Ed.), Plant Nutrition - Physiology and Applications. Kluwer Academic Publishers, Dordrecht, pp. 619–623.

Raitio, H., Paukkonen, K., Kauppi, A., 1994. Effects of defoliation, nitrogen nutrition, and temperature on leafing and root carbohydrates of birch seedlings. Can. J. For. Res. 24, 1914–1920.

Richardson, A.D., Carbone, M.S., Keenan, T.F., Czimczik, C.I., Hollinger, D.Y., Murakami, P., Schaberg, P.G., Xu, X., 2013. Seasonal dynamics and age of stemwood nonstructural carbohydrates in temperate forest trees. New Phytol. 197, 850–861.

Roccuzzo, G., Scandellari, F., Allegra, M., Torrisi, B., Stagno, F., Mimmo, T., Zanotelli, D., Gioacchini, P., Millard, P., Tagliavini, M., 2017. Seasonal dynamics of root uptake and spring remobilisation of nitrogen in field grown orange trees. Sci. Hort. 226, 223–230.

Rosner, S., Kartusch, B., 2003. Structural changes in primary lenticels of Norway spruce over the seasons. IAWA J. 24, 105–116.

Sanders, D., Brownlee, C., Harper, J.F., 1999. Communicating with calcium. Plant Cell 11, 691–706.

Sattelmacher, B., 2001. The apoplast and its significance for plant mineral nutrition. New Phytol. 149, 167–192.

Sevanto, S., Vesala, T., Perämäki, M., Nikinmaa, E., 2003. Sugar transport together with environmental conditions controls time lags between xylem and stem diameter changes. Plant Cell Environ. 26, 1257–1265.

Shear, C.B., Faust, M., 1970. Calcium transport in apple trees. Plant Physiol. 45, 670–674.

Singh, S., 2016. Root pressure: getting to the root of pressure, in: Luttge, U., Canovas, F.M., Matyssek, R. (Eds.), Progress in Botany 77. Springer International Publishing, Switzerland, pp. 105–150.

Stassen, P.J.C., Stadler, J.D., 1988. Seasonal uptake of phosphorus, potassium, calcium and magnesium by young peach trees. South African J. Plant Soil 5, 19–23.

Steppe, K., De Pauw, D.J.W., Lemeur, R., Vanrolleghem, P.A., 2005. A mathematical model linking tree sap flow dynamics to daily stem diameter fluctuations and radial stem growth.

Tree Physiol. 26, 257–273.

Steppe, K., Lemeur, R., 2004. An experimental system for analysis of the dynamic sap flow characteristics in young trees: results of a beech tree. Funct. Plant Biol. 31, 83–92.

Taiz, L., Zeiger, E., Moller, I.M., Murphy, A., 2015. Plant physiology and development, 6th ed. Sinauer Associates, Inc., Sunderland, MA, USA.

Tanner, W., Beevers, H., 2001. Transpiration, a prerequisite for long-distance transport of minerals in plants? Proc. Natl. Acad. Sci. 98, 9443–9447.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Titus, J.S., Kang, S.-M., 1982. Nitrogen metabolism, translocation, and recycling in apple trees. Hort. Rev. 4, 204–246.

Tokarz, K., Pilarski, J., 2005. Optical properties and the content of photosynthetic pigments in the stems and leaves of the apple tree. Physiol. Plant. 27, 183–191.

Tromp, J., 1975. The effect of temperature on growth and mineral nutrition of fruits of apple, with special reference to calcium. Physiol. Plant. 33, 87–93.

Tromp, J., 1983. Nutrient reserves in roots of fruit trees, in particular carbohydrates and nitrogen. Plant Soil 71, 401–413.

Tromp, J., Van Vuure, J., 1993. Accumulation of calcium, potassium and magnesium in apple fruits under various conditions of humidity. Physiol. Plant. 89, 149–156.

Valenzuela-Estrada, L.R., Vera-Caraballo, V., Ruth, L.E., Eissenstat, D.M., 2008. Root anatomy, morphology, and longevity among root orders in *Vaccinium corymbosum* (*Ericaceae*). Am. J. Bot. 95, 1506–1514.

Van de Geijn, S.C., Petit, C.M., 1979. Transport of divalent cations. Cation exchange capacity of intact xylem vessels. Plant Physiol. 64, 954–958.

Van de Geijn, S.C., Smeulders, F., 1981. Diurnal changes in the flux of calcium toward meristems and transpiring leaves in tamato and maize plants. Planta 151, 265–271.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Volder, A., Smart, D.R., Bloom, A.J., Eissenstat, D.M., 2005. Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: implications for root efficiency and competitive effectiveness. New Phytol. 165, 493–501.

Wallace, A., Mueller, R.T., 1980. Calcium uptake and distribution in plants. J. Plant Nutr. 2, 247–256.

Wang, S.Y., Faust, M., Line, M.J., 1994. Apical dominance in apple (*Malus domestica* Borkh): The possible role of indole-3-acetic acid (IAA). J. Amer. Soc. Hort. Sci. 119, 1215–1221.

Wang, H., Inukai, Y., Yamauchi, A., 2006. Root development and nutrient uptake. Crit. Rev. Plant Sci. 25, 279–301.

Wegner, L.H., 2014. Root pressure and beyond: Energetically uphill water transport into xylem vessels? J. Exp. Bot. 65, 381–393.

Wells, C.E., Eissenstat, D.M., 2001. Marked differences in survivorship among apple roots of different diameters. Ecology 82, 882–892.

Wells, C.E., Eissenstat, D.M., 2003. Beyond the roots of young seedlings: The influence of age and order on fine root physiology. Plant Growth Regul. 21, 324–334.

White, P.J., 1998. Calcium channels in the plasma membrane of root cells. Ann. Bot. 81, 173–183.

White, P.J., 2001. The pathways of calcium movement to the xylem. J. Exp. Bot. 52, 891–899.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilcox, H.E., 1968. Morphological studies of the root of red pine, *Pinus resinosa.* I. Growth characteristics and patterns of branching. Am. J. Bot. 55, 247–254.

Yoshioka, H., Nagai, K., Aoba, K., Fukumoto, M., 1988. Seasonal changes of carbohydrates metabolism in apple trees. Sci. Hort. 36, 219–227.

Zhang, H., Rong, H., Pilbeam, D., 2007. Signalling mechanisms underlying the morphological responses of the root system to nitrogen in *Arabidopsis thaliana*. J. Exp. Bot. 58, 2329–2338.

Zweifel, R., Item, H., Häsler, R., 2000. Stem radius changes and their relation to stored water

in stems of young Norway spruce trees. Trees 15, 50–57.

Zweifel, R., Item, H., Häsler, R., 2001. Link between diurnal stem radius changes and tree water relations. Tree Physiol. 21, 869–877.

Table 1. Main stem diameter (mm) and tree height (m) of two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 in response to $Ca(NO_3)_2$ soil applications in May 2016, after consideration of the covariate (initial measurements at planting in September 2015).

Treatment	Diameter (mm)	Length (m)
Natural leaf drop (NLD)		
Control (None)	16.87 ns	0.96 ns
Moderate (1X) [a]	16.74	0.93
High (2X) [b]	16.88	0.98
Autumn defoliation (AD)		
High (2X) [c]	16.21	1.03
P-value	0.7734	0.6035

Adjusted means followed by "ns" are not significantly different at $P \leq 0.05$.

[a] 75 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[c] 150 g $Ca(NO_3)_2$ pot^{-1} after leaf removal in April 2016.

Table 2. Fresh mass (g) and dry mass (g) of the roots, stems (two-year-old wood), shoots (one-year-old wood including spurs) and leaves of two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 in response to $Ca(NO_3)_2$ soil applications in May 2016.

Treatment	Fresh mass (g)				Dry mass (g)			
	Roots	Stems	Shoots	Leaves [d]	Roots	Stems	Shoots	Leaves [d]
Natural leaf drop (NLD)								
Control (None)	407.43 a	246.41 ns	73.13 a	(20.17)	148.36 a	126.56 ns	33.46 a	(7.08)
1X (Moderate) [a]	344.71 ab	231.31	64.38 a	(31.88)	145.70 ab	118.62	28.88 a	(21.21)
2X (High) [b]	322.40 b	233.80	63.83 a	(61.58)	124.43 b	115.10	27.25 a	31.04 b
Autumn defoliation (AD)								
2X (High) [c]	200.83 c	215.70	38.25 b	(94.00)	74.58 c	102.51	14.63 b	36.96 a
P-value	< 0.0001	0.6103	0.0026		< 0.0001	0.2054	0.0005	0.0454

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 75 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[c] 150 g $Ca(NO_3)_2$ pot^{-1} after leaf removal in April 2016.

[d] Differences in leaf FM between treatments, as well as leaf DM between the control and 1X treatment, was not analysed statistically. In the control and 1X treatment, most leaves were lost during natural leaf drop, whereas the leaves recovered from the $2X_{(NLD)}$ treatment at the end of August included all abscised leaves that were collected in nets from the onset of leaf drop plus the leaves that were still firmly attached to the trees at the end of August. In the $2X_{(AD)}$ treatment, all leaves were fresh and still firmly attached to the trees prior to defoliation earlier in autumn (before the onset of NLD). Drying of all leaf samples of the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments, however, allowed for a comparison in leaf DM between these two treatments.

Table 3. Calcium concentration (% DM) of the roots and stems of the control trees at planting (September 2015). Calcium and nitrogen concentrations (% DM) of the roots, stems (two-year-old wood), shoots (one-year-old wood including spurs) and leaves of two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 in response to $Ca(NO_3)_2$ soil applications in May 2016.

Treatment	Calcium (% DM)				Nitrogen (% DM)			
	Roots	Stems	Shoots	Leaves	Roots	Stems	Shoots	Leaves
Control (at planting)	0.22 b	0.53 ns						
Natural leaf drop (NLD)								
Control (None)	0.24 b	0.48	0.76 ns	1.49 b	0.98 c	1.31 b	1.42 b	1.75 b
1X (Moderate) [a]	0.24 b	0.47	0.67	1.36 b	1.59 b	1.70 a	2.11 a	2.18 a
2X (High) [b]	0.25 b	0.38	0.71	1.76 a	1.89 a	1.70 a	2.22 a	2.00 a
Autumn defoliation (AD)								
2X (High) [c]	0.38 a	0.42	0.75	1.03 c	1.45 b	1.16 b	1.38 b	2.20 a
P-value	0.0012	0.1578	0.6977	<0.0001	< 0.0001	0.0004	< 0.0001	0.0007

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 75 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[c] 150 g $Ca(NO_3)_2$ pot^{-1} after leaf removal in April 2016.

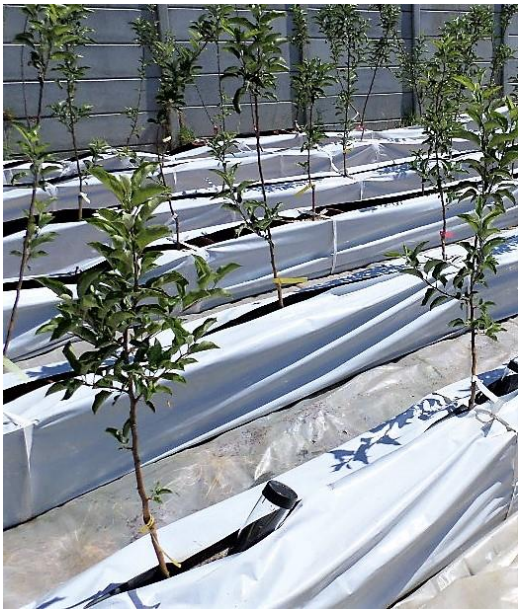


Fig. 1. Two-year-old, potted ‘Golden Delicious’/M7 apple trees as placed on a transparent plastic tarp, evenly spaced in rows, with an additional sheet of white plastic tarp strapped around the base and sides of the pots along each individual row. The exposed end of a minirhizotron tube (80 mm diameter x 0.5 m length) that was inserted at a 45° angle from the vertical in the soil, parallel to the work row, with the base slanted away from the stem, is visible.

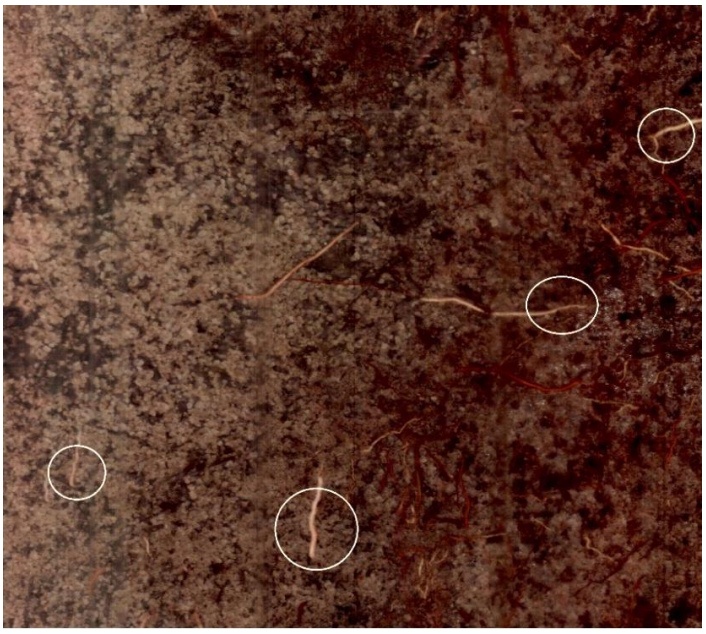


Fig. 2. Minirhizotron image (21.6 x 19.6 cm window) of the root zone of a single, two-year-old, potted, non-bearing ‘Golden Delicious’/M7 apple tree that received high $Ca(NO_3)_2$ (2X) soil applications in May 2016, prior to the onset of leaf drop mid-May 2016. Roots marked with white circles are examples of white root tips that were manually counted to quantify white root growth at that time. Brown, woody and blackened roots were excluded from the counts.

Fig. 3. Light-weight net (Netlon Netting, SA) draped over the canopy of a two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple tree on 5 May 2016, that received high $Ca(NO_3)_2$ (2X) soil applications. All absisced leaves were recovered from the start to the end of leaf drop (mid-May 2016 to end of August 2016).

(a) (b)

Fig. 4. Single dendrometers installed on 14 April 2016, around the main stems of two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees that received high $Ca(NO_3)_2$ (2X) soil applications, where (a) leaves were left to drop naturally (NLD) or (b) all leaves were removed by hand on 22 April 2016 (AD).

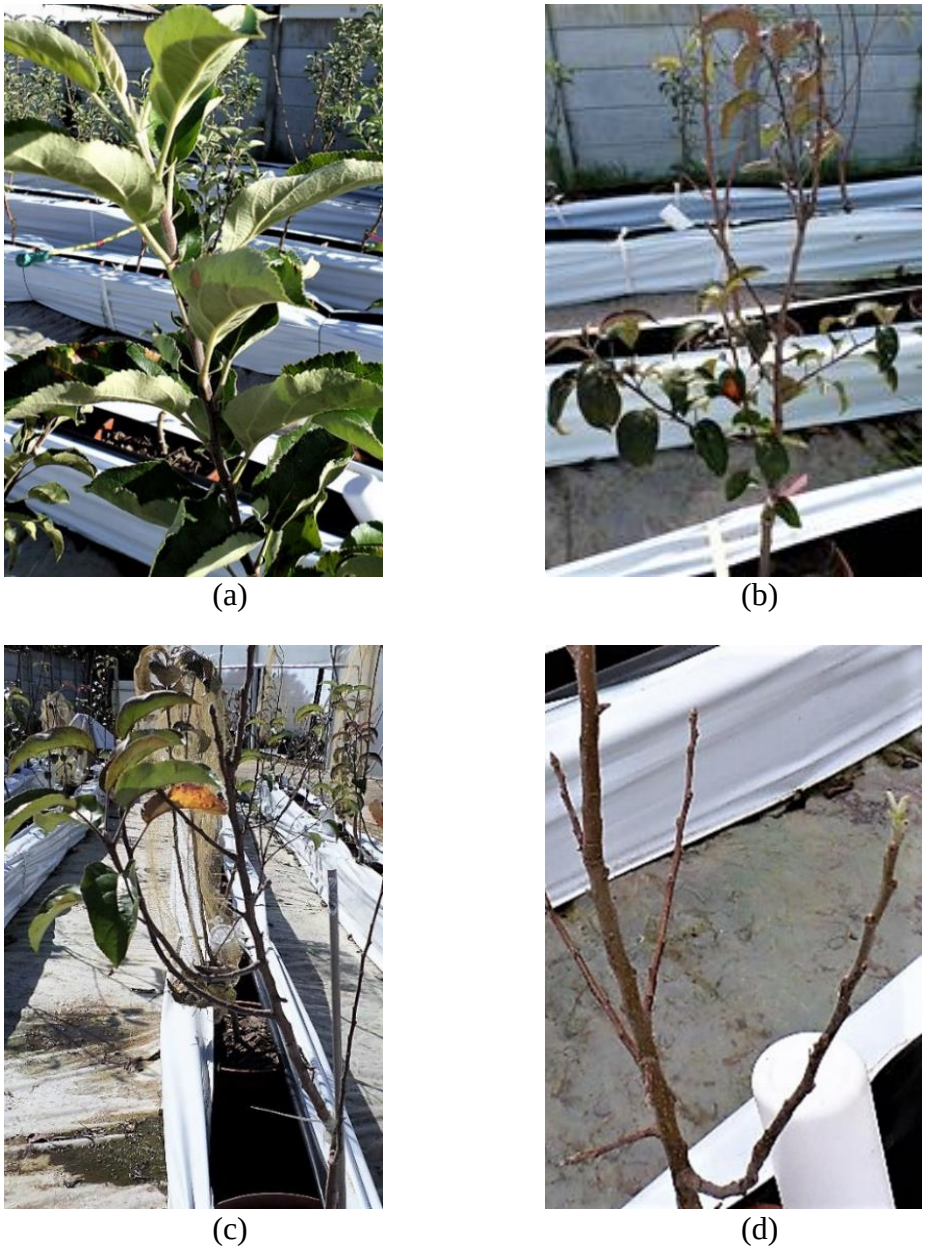


Fig. 5. Photographic evidence of various stages of tree phenology of two-year old, potted, non-bearing ‘Golden Delicious’/M7 apple trees during autumn/winter, where (a) shows re-flushing of the current season’s shoots during April 2016, (b) 50 % leaf drop in July 2016, (c) 90 % leaf drop towards the end of August 2016, and (d) bud burst during May 2016 in autumn-defoliated (AD) trees.

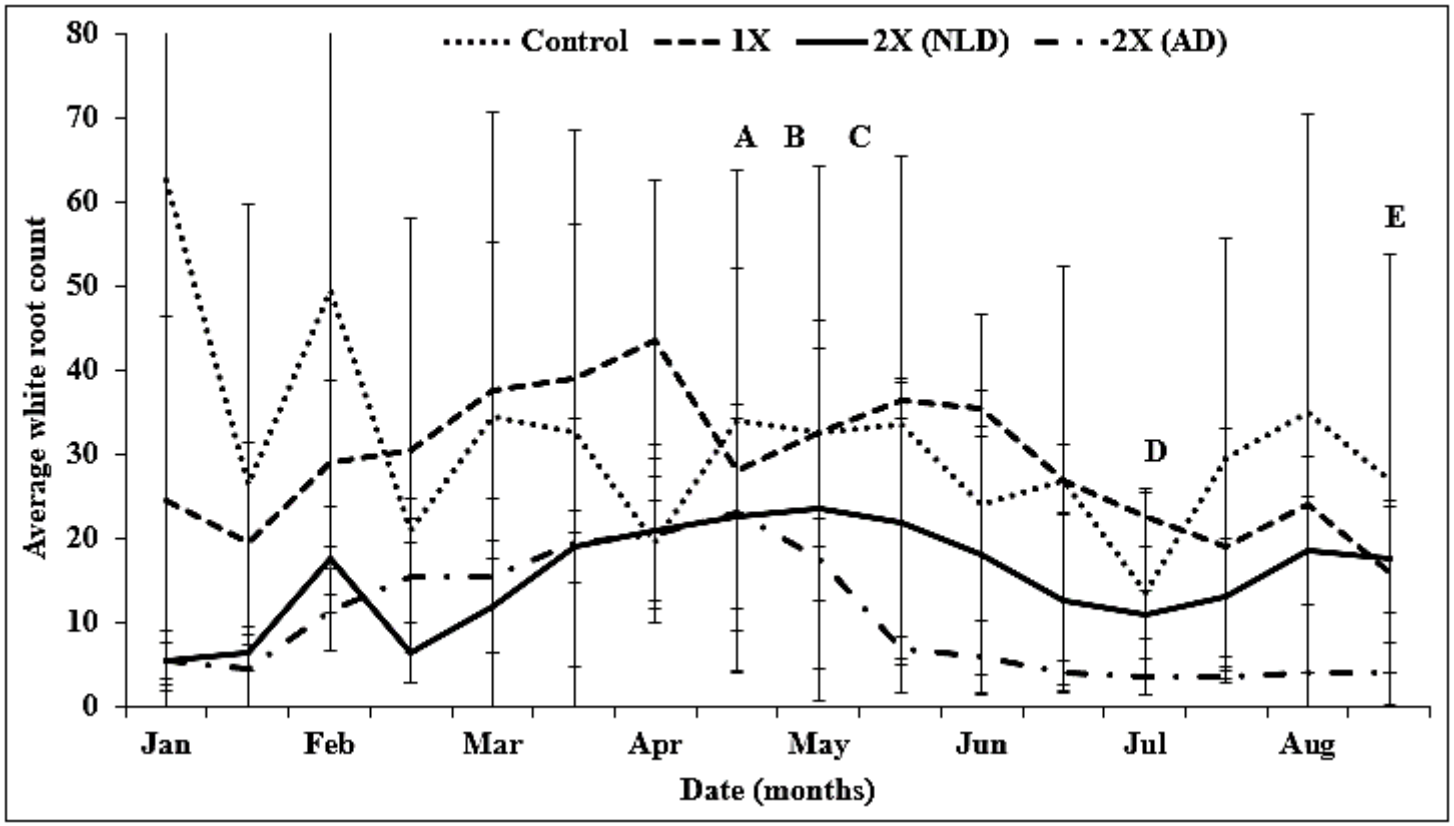


Fig. 6. Average white root counts of two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees recorded from January to end of August 2016 for the control, moderate $Ca(NO_3)_2$ (1X) and high $Ca(NO_3)_2$ (2X) treatments that experienced natural leaf drop (NLD) and the high $Ca(NO_3)_2$ (2X) treatment that was subjected to complete defoliation in autumn (AD), where (A) represents execution of AD, (B) commencement of $Ca(NO_3)_2$ soil applications, (C) onset of leaf drop, (D) 50 % leaf drop, and (E) 90 % leaf drop. Vertical bars denote standard errors of the mean counts of two replications per treatment.

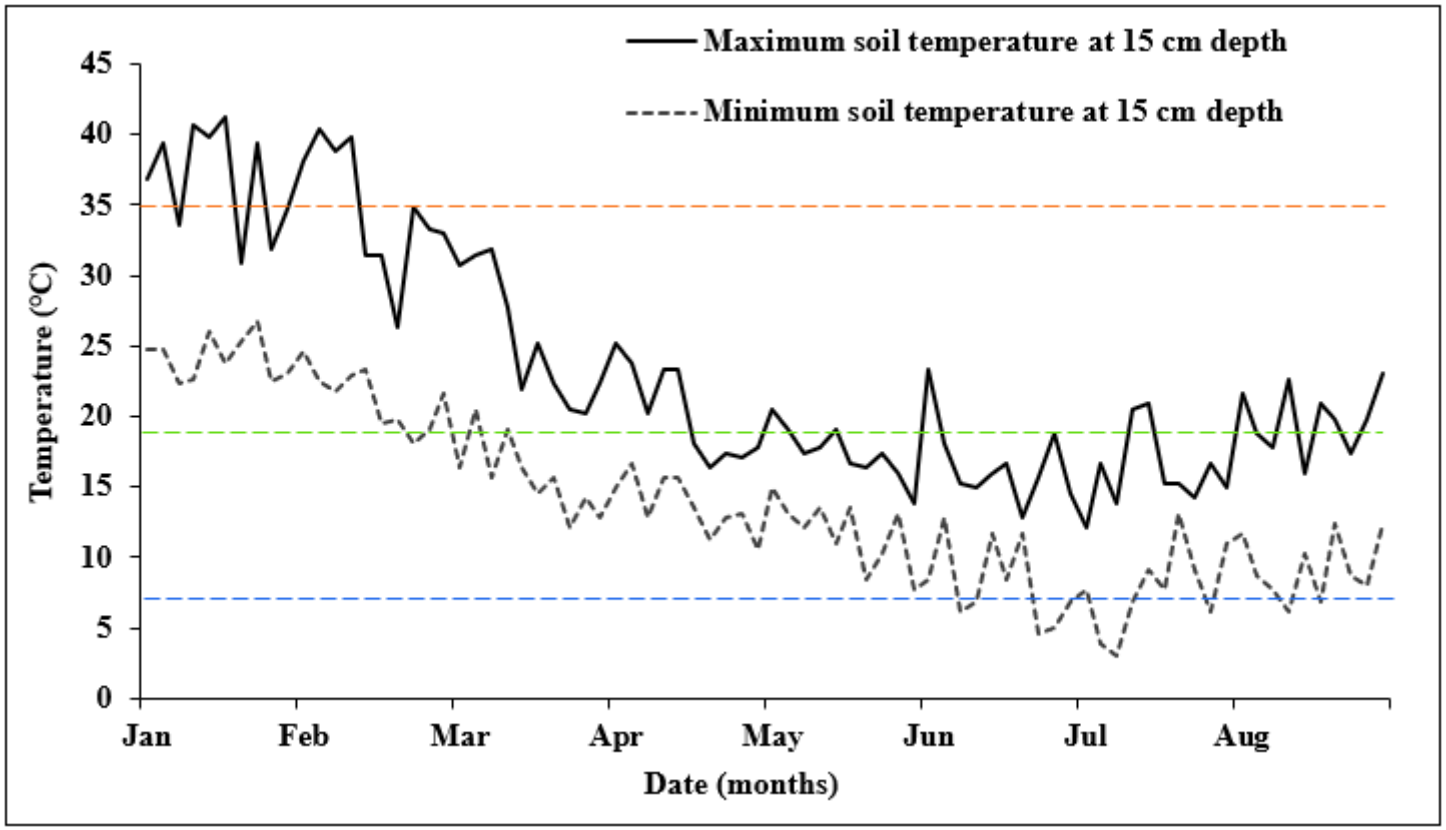
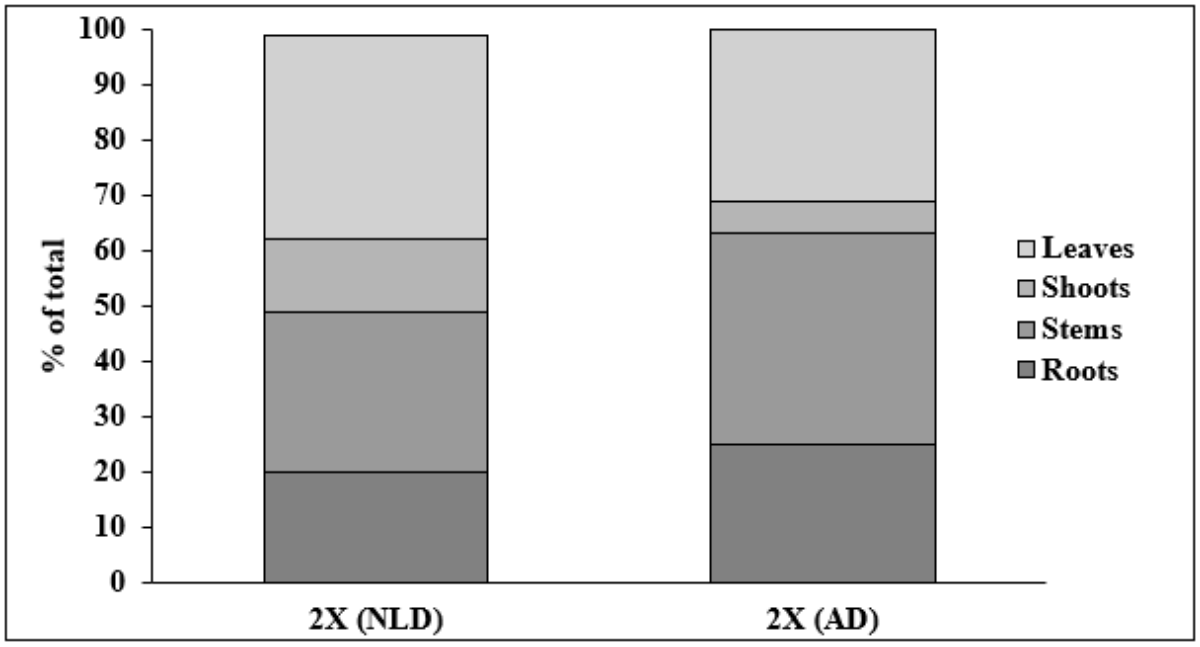
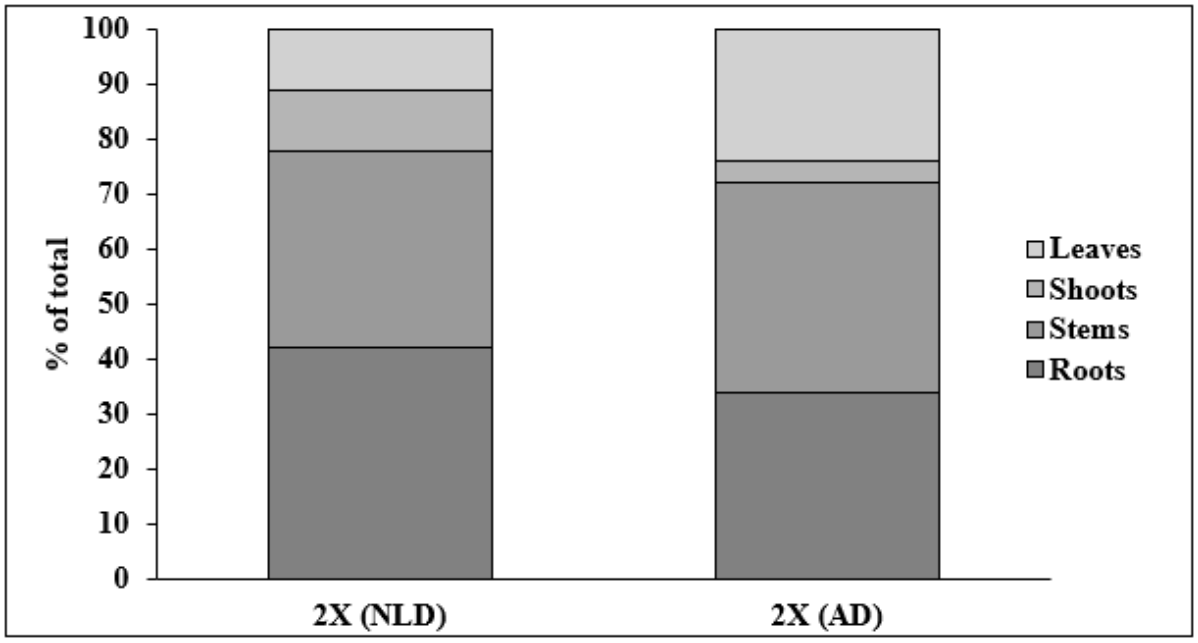


Fig. 7. Soil temperature profile in the pots at a depth of 15 cm recorded on the hour with Tiny-tag soil probes (TPG-4505 Gemini Data Loggers Ltd., Chichester, West Sussex, UK) for the period January to end-August 2016. The green line depicts the optimum root growth temperature, the red line the maximum temperature for root growth, and the blue line the minimum temperature for root growth (Nightingale, 1935).



Treatment	Roots	Stems	Shoots	Leaves
$2X_{(NLD)}$	20 b	29 b	13 a	37 a
$2X_{(AD)}$	25 a	38 a	6 b	31 b
P-value	0.0455	0.0108	0.0035	0.0024

Fig. 8. Partitioning (% of total) of Ca content within two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 for the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments, where $2X_{(NLD)}$ = 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016, prior to the onset of leaf drop mid-May 2016, and $2X_{(AD)}$ = 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016, after leaf removal in April 2016. Percentage of total Ca content in each plant part = (Ca content (g DM) in each respective plant part (Annexure 1, Table 2))/(Total Ca content (g DM) per tree) × 100. Means with different letters are significantly different at $P \leq 0.05$.



Treatment	Roots	Stems	Shoots	Leaves
$2X_{(NLD)}$	42 a	36 ns	11 a	11 b
$2X_{(AD)}$	35 b	38	4 b	24 a
P-value	0.0036	0.1935	0.0004	< 0.0001

Fig. 9. Partitioning (% of total) of N content within two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 for the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments, where $2X_{(NLD)}$ = 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016, prior to the onset of leaf drop mid-May 2016, and $2X_{(AD)}$ = 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016, after leaf removal in April 2016. Percentage of total N content in each plant part (% of total) = (N content (g DM) in each respective plant part (Annexure 1, Table 2))/(Total N content (g DM) per tree) × 100. Means with different letters are significantly different at $P \leq 0.05$.

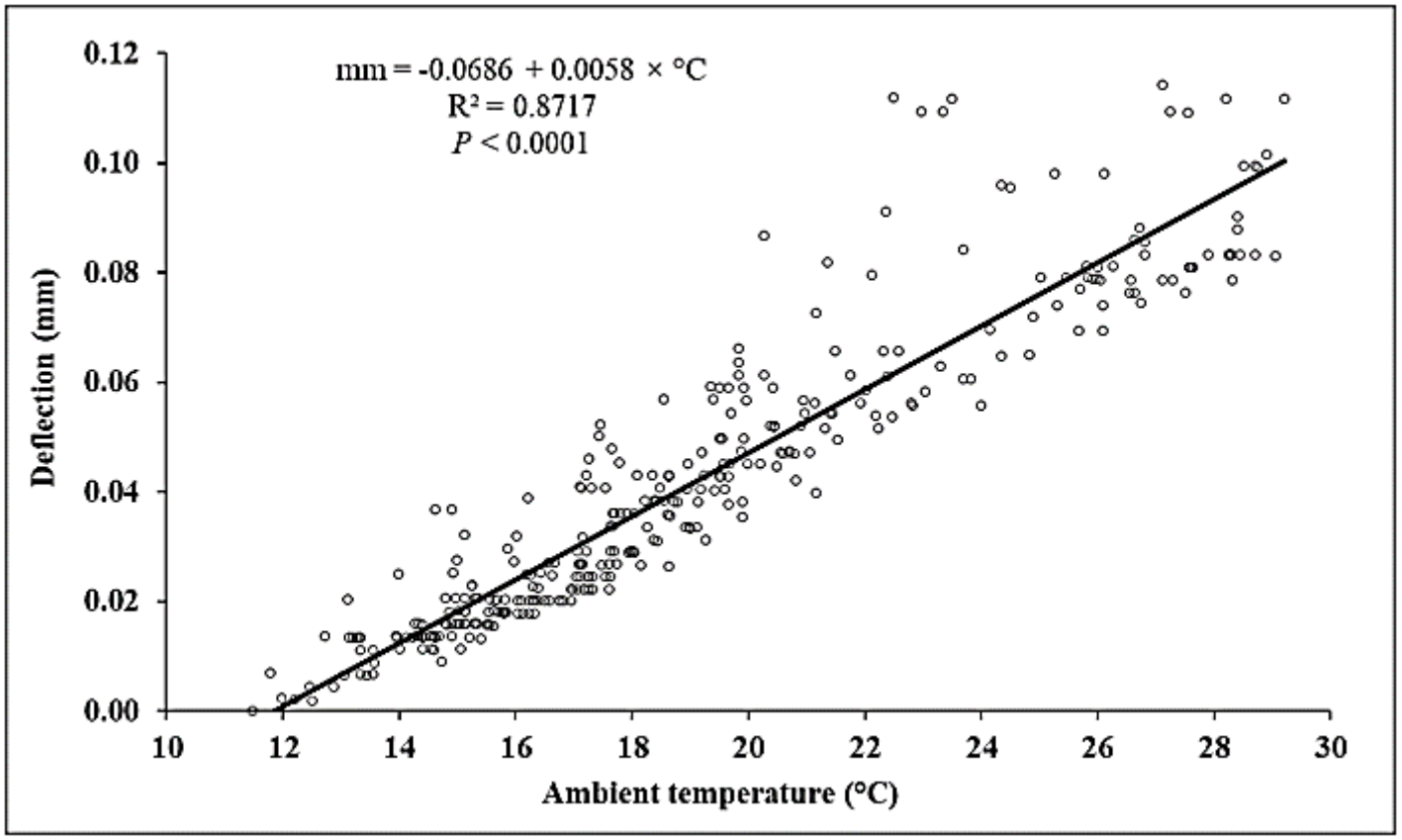


Fig. 10. Relationship between ambient temperature (°C) and DEX70 (Dynamax Inc., Houston, TX, USA) dendrometer deflections (mm) on a PVC cylinder that was secured in an upright position at the experimental site.

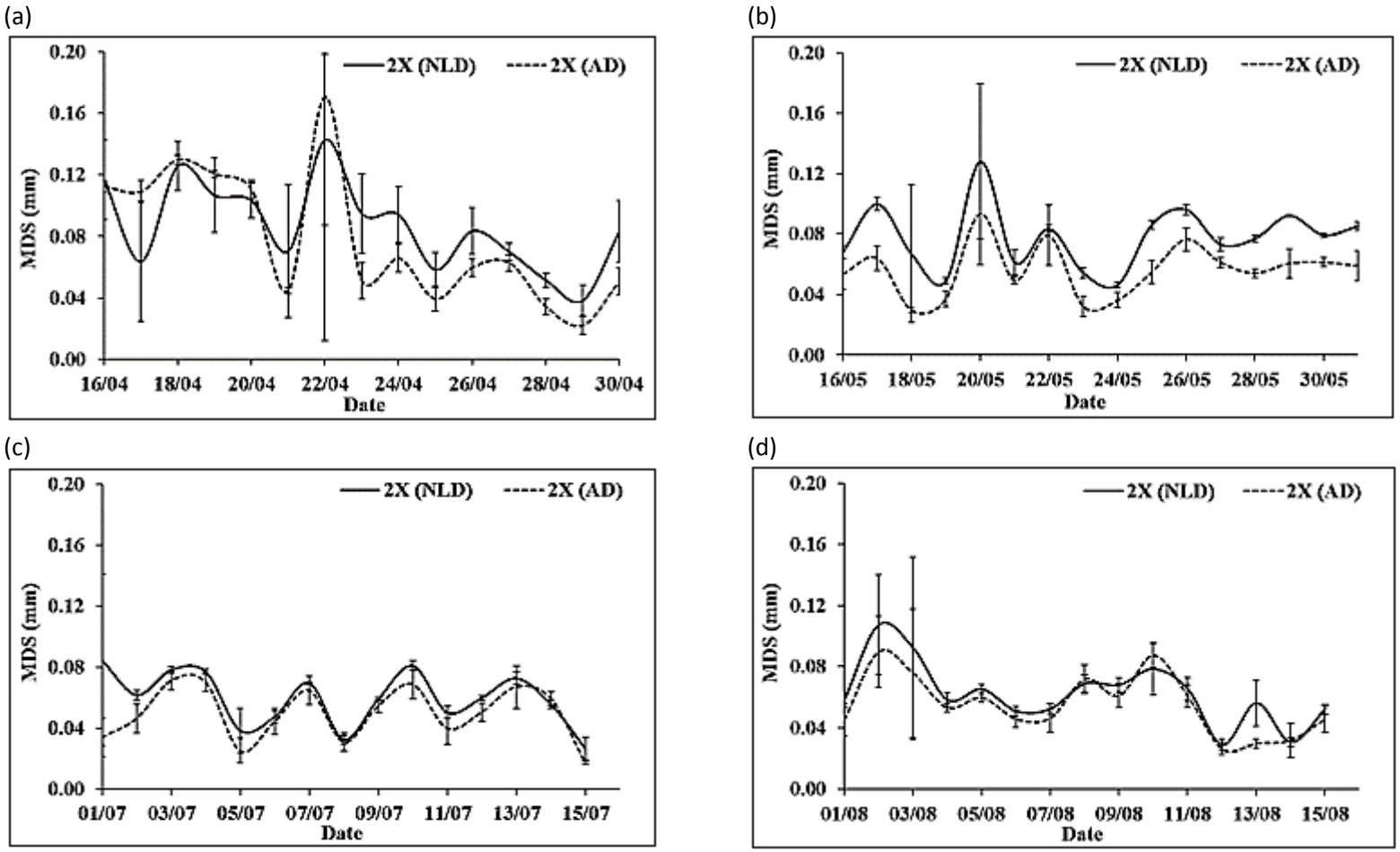


Fig. 11. Maximum daily stem shrinkage (MDS) of two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees between high $Ca(NO_3)_2$ (2X) treatments that experienced natural leaf drop (NLD) and were subjected to autumn defoliation (AD), recorded in April (a), May (b), July (c), and August 2016 (d). Defoliation of trees took place on 22 April 2016. The onset of leaf drop was noted mid-May and 50 % leaf drop, mid-July 2016. Data points are the mean ± SE of three replications per treatment.

PAPER 2

Quantifying calcium uptake and distribution in apple root tips by scanning electron microscopy and wavelength-dispersive x-ray spectroscopy: A pilot study

Abstract

In previous experiments conducted on apple trees (*Malus domestica* Borkh.) in various apple-growing regions of the Western Cape, soil-applied calcium nitrate ($Ca(NO_3)_2$) synchronized with a period of active white root growth in autumn, markedly increased the Ca concentration of the roots and reserve tissues (wood and bark) of the trees. However, these findings only provided an indication of total root Ca concentration per weight, with no distinction being made between white, absorptive roots and brown or woody roots. Their respective contributions to total Ca uptake in apple are, therefore, not known. Since young white roots or root tips have the highest potential for soil Ca uptake, quantifying Ca uptake and distribution in apple root tips in relation to above-ground tree phenology during autumn/winter could improve our understanding of root Ca uptake and translocation processes during dormancy. Chemical analysis to determine the Ca content in root tissues requires the destruction of a substantial amount of plant material, thus, preventing in situ determinations of Ca concentration in specific locations along the length of individual roots. Scanning electron microscopy (SEM) and wavelength-dispersive x-ray spectroscopy (WDS) may provide this information by combining Ca localization and quantification in white root segments with digital images. In this pilot study, tissue Ca concentration and distribution along the length of apple white root tips at set time intervals during winter following soil applications of $Ca(NO_3)_2$ towards the end of autumn were determined using SEM-WDS. This technique proved useful in determining in situ concentration and distribution in apple white root tips during winter, yet, the digital images did not provide the necessary information on root endodermal development to distinguish between the apoplastic and symplastic pathways of Ca movement to the xylem with certainty. Overall, observations suggest rapid uptake of soil-applied Ca by apple white root tips during winter due to an extended leaf drop period. Although root Ca concentrations did not differ significantly between trees of the control, which received no additional Ca, and trees of the Ca treatment, which received a relatively high concentration of Ca via soil applications of $Ca(NO_3)_2$,

observations suggest a differential pathway of Ca translocation to the shoot before and after 50 % leaf drop. The possibility that the observed Ca accumulation patterns arose from differences in root growth rates and root anatomical development is discussed in relation to tree phenology and shoot demand.

Key words: Apoplast, Casparian band, hexamethyldisilazane (HMDS), symplast, calcium translocation.

1. Introduction

At almost any point during the season, the perennial root system of fruit trees comprise a heterogenous population of roots that vary in type, age, lifespan and physiological function (Wells and Eissenstat, 2003; Hishi, 2007; Guo et al., 2008; Zadworny and Eissenstat, 2011; Rewald et al., 2014; McCormack et al., 2015, 2017). All newly produced roots are initially white in colour and turn brown with age several weeks to months later, depending on the time of season (Nightingale, 1935; Head, 1966; Atkinson, 1983). Natural root browning is associated with condensed tannin deposition in the walls of collapsed root cortical cells, which proceeds from the older basal region of the root towards the tip (Richards and Considine, 1981; Mckenzie and Peterson, 1995a; McCrady and Comerford, 1998; Peterson et al., 1999). Primary brown roots become woody as they shed their cortex and undergo secondary growth to increase in diameter and develop a cork zone (Richards and Considine, 1981; Mckenzie and Peterson, 1995b; McCrady and Comerford, 1998; Peterson et al., 1999).

When passing from white to brown (primary) to woody (secondary), roots undergo marked changes in physiological function. One such change is a shift in nutrient uptake potential (Wells and Eissenstat, 2003; Hishi, 2007; Baldi et al., 2010; Comas et al., 2010). Although numerous studies have shown that brown roots are viable and capable of water and nutrient uptake (Crider, 1933; Atkinson and Wilson, 1979, 1980; Escamilla and Comerford, 2000), their capacity in this regard tends to decrease because of changes in their anatomical structure. These changes include the development of apoplastic barriers to water and nutrient uptake, the loss of epidermal and root cortical tissues resulting in a reduction in plasmalemma surface area available for uptake, and qualitative and quantitative changes in membrane proteins (e.g. channels, carriers and pumps) that direct water and nutrient uptake (Kamula et al., 1994; Mckenzie and Peterson, 1995a, b; Peterson et al., 1999; Schreiber et al., 1999; Enstone et al.,

2003; Gu et al., 2015). Since brown roots comprise the greatest surface area of tree root systems during most of the growing season, their contribution to total nutrient uptake should not be dismissed (Atkinson and Wilson, 1979; Comerford et al., 1994; Hawkins et al., 2014). However, the higher nutrient uptake potential of young white roots or root tips (Clarkson, 1984, 1993; Bouma et al., 2001; Volder et al., 2005; De Freitas and Mitcham, 2012; Gu et al., 2015) could mean meeting the tree nutrient demand during seasonal peaks of white root production.

In a potting trial, Van Zyl (2016) found that soil applications of a recommended standard rate of calcium nitrate (8 g $Ca(NO_3)_2$ 5 L pot^{-1}) in autumn was sufficient to markedly increase the Ca concentration of the roots and reserve tissues (wood and bark) of young non-bearing 'Golden Delicious'/M7 apple trees three weeks after soil application. This study provided important preliminary information for a better understanding of allocation patterns of soil-applied Ca to the roots, stems and new growth of locally grown apple trees in autumn, however, no distinction was made between newly produced white roots or white root tips, and older, brown or woody roots. In another experiment, Van Zyl (2016) observed an extended period of white root production and growth in mature apple trees during autumn and into winter. White root numbers increased in March, peaked in June, and gradually decreased towards the end of August. Since various authors have shown that young white roots or root tips have a higher potential for soil nutrient uptake, the observations by Van Zyl (2016) encouraged an interest in the dynamics of Ca uptake and distribution along the lengths of apple root tips during winter. Despite the importance of understanding the dynamics of uptake, translocation and storage of nutrients such as Ca in the fine roots during fruit tree dormancy (Terblanche, 1972; Terblanche et al., 1979; Ferguson, 1980; Majdi et al., 2001), this has not been fully explored.

Much useful information regarding this subject has been obtained from studies that employed the following techniques: (1) exposure of sequential root segments of intact bean, maize and marrow roots to labelled nutrients, e.g. ^{45}Ca (Biddulph, 1967; Harrison-Murray and Clarkson, 1973; Ferguson and Clarkson, 1975, 1976), (2) measurement of ion fluxes across the root surface of maize seedlings with ion-specific microelectrodes (Ryan et al., 1990), (3) investigation of major pathways of Ca^{2+} transport in onion roots by membrane protein inhibitors, e.g. lanthanum (La^{3+}) and vanadate (VO_4^{3-}) (Cholewa and Peterson, 2004) and (4) determination of ion localization in roots of maize, soybean and rice seedlings by scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDS) (Chino and Baba, 1976; Chino and Hidaka, 1977; Chino, 1979). Results from these studies showed that Ca

translocation from the apical 5 to 15 mm of the roots of all studied species were fairly similar. In this region, xylem vessels were immature and Casparian bands in the endodermis were mostly absent. Marked differences in uptake capacity were found in the more basal regions of the roots. In marrow, barley, maize and onion roots, the deposition of Casparian bands (> 15 mm from the tip) and the subsequent suberization of the endodermal cells (> 80 mm from the tip) acted as a significant apoplastic barrier for Ca transport, indicated by the significantly higher accumulation of Ca at the endodermis compared to the stele, which now had functional xylem, whereas in bean and soybean roots, Ca was shown to be readily translocated into the stele despite these developments. Chino (1979) suggested that this may be due to the higher permeability of the endodermis to Ca in the roots of bean plants. Although the results of these studies provide a useful guide to our investigation of soil-applied Ca uptake and distribution along the lengths of apple root tips during winter, this data may have limited relevance to soil-grown apple roots of potted trees under field conditions. Deductions were based on uptake by young, white seminal and lateral roots of crop seedlings grown in solution culture, and in most cases, determinations were made within 24 hours after transfer to the absorption medium. In addition to species variation, roots grown in solution culture under controlled conditions may exhibit marked differences in anatomical development compared to roots grown in the field (Enstone et al., 2003; Kumar et al., 2007; Song et al., 2011). Indeed, the sequential stages of endodermal development in soil-grown apple roots tend to occur much closer to the tip, i.e. State I development occurs within 3 – 5 mm from the apex, State II development, after 16 mm from the apex, and state III development, after 30 mm from the apex (Mackenzie, 1979). State I refers to the deposition of lignified Casparian bands within the transverse and radial longitudinal (anticlinal) walls of the endodermal cells to which the plasma membrane of the cells become firmly attached, State II to the deposition of suberin lamellae on the inner walls of most but not all endodermal cells (the latter termed, “passage cells” that remain in state I), and State III to cell wall thickening by further deposition of suberin, followed by the deposition of lignified, carbohydrate or cellulosic cell walls internal to the suberin lamellae of state II endodermal cells (Mackenzie, 1979; Peterson and Enstone, 1996; Schreiber et al., 1999; White, 2001; Geldner, 2013; Meyer and Peterson, 2013).

Multiple orders of lateral fine roots develop from the primary root axes of an expanding tree root system; the roots within each lateral fine root branch displaying great variability in morphology, anatomy and physiological activity (Atkinson, 1983; Eissenstat and Achor, 1999; Wells and Eissenstat, 2003; Polverigiani et al., 2011; Zadworny and Eissenstat, 2011;

Bagniewska-Zadworna et al., 2012). In comparison with the majority "short", "fibrous" or "absorptive" roots; "long", "pioneer" or "extension" roots are larger in diameter and extend more rapidly and indeterminately into the soil from the time of their initiation. Moreover, faster growing roots tend to develop endodermal Casparian bands further from the tip compared to slower growing roots (Wilcox, 1962; Ferguson and Clarkson, 1975; Waisel and Eshel, 2002; Enstone et al., 2003); the former allowing less selective nutrient uptake via the apoplast at an increased distance from the tip in contrast to the latter, where Casparian band deposition closer to the tip dictates symplastic flow across the endodermis, allowing roots to control the rate and selectivity of Ca transport to the shoot (Clarkson, 1984, 1993; White, 1998, 2001; White and Broadley, 2003; Wang et al., 2006).

To date, soil-applied Ca uptake and distribution along the length of apple white root tips during winter has not been investigated or quantified. To address this knowledge gap, we used ambient-SEM along with wavelength-dispersive x-ray spectroscopy (WDS) to quantify Ca uptake and distribution in apple white root tips harvested from young, potted, non-bearing 'Golden Delicious'/M7 apple trees grown in control (no additional soil-applied Ca) and Ca treated soil during winter. Although destructive tissue analysis can provide useful information with regards to Ca uptake, it requires large amounts of plant tissue and it does not allow discrete analysis of localized areas in the tissue. Scanning electron microscopy along with x-ray microanalysis has been shown to be a reliable technique for the study of nutrient element localization and quantification in plant tissues (Storey and Leigh, 2004; Cocozza et al., 2008; Hunsche and Noga, 2008). To preserve the original form and structure of plant tissues as close as possible to their natural state for proper analysis, tissue preparation entails several steps, including chemical fixation, dehydration and drying. However, as plants constitute a broad variety of tissue types that differ in form, structure and composition, a universal SEM preparation technique is difficult (Pathan et al., 2008; Clode, 2015). It is, therefore, imperative to develop and test specific techniques for specific tissue types (Pathan et al., 2008). An ancillary objective was, thus, to find a suitable and cost-effective tissue preparation technique for SEM-WDS analysis of young, white root tips.

Our main objective in this pilot study was to quantify Ca uptake and distribution along the length of apple white root tips during winter following late-autumn soil $Ca(NO_3)_2$ applications during a period of active white root growth under local conditions.

2. Materials and Methods

2.1. Plant material and growth conditions

This experiment was performed on selected trees at the Welgevallen Experimental Farm (33°56'33"S, 18°51'56"E) of Stellenbosch University in the Western Cape that formed part of a larger experiment focusing on soil Ca applications during a period of active white root growth in autumn and into winter in two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees (Paper 1).

Trees were fertigated daily with a low Ca, balanced nutrient solution through an automatically controlled drip irrigation system, described in full in Paper 1. In May 2016 (late-autumn), individual trees were randomly assigned to two groups of treatments – soil applications of $Ca(NO_3)_2$ (YaraLiva Nitrabor®, Yara Africa Fertilizer (Pty). Ltd.) comprising a relatively high concentration of Ca (150 g 25 L pot^{-1} split applied by hand in equal quantities on a weekly basis over a three-week period to the surface of each pot = $2X_{(NLD)}$ treatment, Paper 1) and a control, that received no additional Ca. Application dates were spaced weekly apart as follows: 6, 13 and 20 May 2016.

2.2. Tree phenology

To relate the dynamics of soil Ca uptake by apple root tips to above-ground tree phenology during autumn/winter, the following phenological growth stages were visually determined and documented: onset of leaf drop, 50 % leaf drop and 100 % leaf drop.

2.3. Microscopy study

2.3.1. Harvesting of roots

Three individual white pioneer roots of uniform size (about 1.5 mm in diameter) and at similar depths per tree were carefully excavated by hand from a larger root mass (Fig. 1). Pioneer roots were identified by their morphology i.e. larger diameter and prominent root tips (Atkinson, 1983; Polverigiani et al., 2011, 2014) and selected because they are less brittle and easier to handle compared with smaller diameter, fibrous roots. Each individual root was cut at approximately 30 mm from the apex and immediately plunged in a solution of 2.5 %

glutaraldehyde (*v*/*v*) in 0.1 M phosphate buffer (pH 7.2). Vials were sealed and stored in a refrigerator at 4 °C for no more than 3 months until processing. Harvesting of roots took place on 10 June 2016, 21 June 2016, 7 July 2016, 10 August 2016 and 25 August 2016, which were 3, 5, 7, 11 and 13 weeks, respectively, after the last soil Ca applications in May 2016. For both treatments, white roots were harvested from alternating trees on each sampling date to prevent excessive disturbance to the root system.

2.3.2. Root preparation

To find a suitable and cost-effective tissue preparation technique for SEM-WDS analysis, six procedures comprising a combination of standard tissue preparation techniques sourced from literature were evaluated (Table 1).

Roots were removed from the glutaraldehyde solution, rinsed in distilled water and individually cross-sectioned with a sharp blade by hand into two separate segments: an apical segment (0 – 5 mm from the root apex) and a basal segment (15 – 20 mm from the root apex). The transverse surface at 5 mm from the apex (apical segment) and at 20 mm from the apex (basal segment) of each root tip was used to measure Ca uptake and distribution along its longitudinal axis. Root segments were fixed in a solution of 2.5 % glutaraldehyde (*v*/*v*) in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h, rinsed in fresh buffer (×3) and dehydrated in an ascending series of ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each). Afterward, individual segments were placed on glass slides and immersed in 100 % HMDS for 3 min immediately after the 100 % ethanol step by applying 3 × 1 ml drops of HMDS to each root segment in 1 min successions. The segments were then allowed to dry in air until evaporation of the organic compound was complete. This process took about 25 – 30 min and was carried out in a laminar flow hood at room temperature (± 22 °C). After the drying stage, each individual segment was mounted on an aluminium stub covered with double-sided, adhesive, energy conductive carbon tape (Agar) and oriented so that the transverse surfaces at 5 mm from the apex (apical segment) and 20 mm from the apex (basal segment) faced upwards. The mounted segments were coated with a 15 nm thick layer of carbon in a Q150T E Turbo-Pumped Carbon Coater (Quorum Technologies Ltd., UK) to ensure good electrical conductivity before they were transferred to the SEM chamber for semi-quantitative mineral analysis.

2.3.3. SEM-WDS analysis

The transverse surfaces of the apical and basal segments of each of three randomly selected white root tips per treatment per sampling date were analysed by quantitative EDS and WDS using a scanning electron microscope (Carl Zeiss SMT (Ltd). EVO MA 15, ZEISS, Germany) at the Central Analytical Facilities (CAF) laboratory of Stellenbosch University. The concentration of constitutive elements i.e. carbon (C), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), sulphur (S), sodium (Na) and silica (Si) determined via EDS using an Oxford Instruments® X-max 20 mm^2 detector were not considered in this study. Calcium was selectively quantified via WDS analysis using an Oxford Instruments® Wave-Dispersive X-ray Spectrometer. High-resolution backscatter images and analytical data were recorded with Oxford INCA software (Oxford Instruments, UK). Beam conditions during analyses were kept constant at a relatively low accelerating voltage of 20.0 kV and approximately 1.0 A to minimize specimen damage during beam exposure, with a sample current of -20.0 nA and a working distance of 8.5 mm. A 1500 × magnification (analysed area ± 1000 μm^2) was used to determine the Ca concentration of several cells at one time. The total time required for each spot analysis was 30 s. Internal Astimex Scientific mineral standards were used for standardization and verification of the analyses.

2.4. Experimental design and data analyses

The experimental layout was a completely randomized four-factor design. Calcium treatments included a control and high Ca treatment. At five respective time-points i.e. 3, 5, 7, 11 and 13 weeks after the last soil applications in May 2016, each experimental unit was divided into an apical and basal segment. Data acquisition was performed on each root cross-section (transverse surface at 5 mm from the apex and transverse surface at 20 mm from the apex) at two areas of interest: xylem vessels in the stele (central cylinder) and parenchyma cells in the mid-cortex (Fig. 2). At each location, the Ca concentration (weight %) was measured at three randomly selected points not more than 100 µm apart to represent each area of interest (Fig. 3). Data were averaged on a location basis and BoxCox transformed to achieve normality. Data were analysed as a completely randomized four-way mixed-level (5 × 2 × 2 × 2) design using StatSoft Statistica software (version 13.2, StatSoft, Inc.). Analysis of variance (ANOVA) was carried out to determine significant differences between the main effects of time (weeks after treatment), treatment, segment (apical and basal), location (stele and cortex) and interaction

terms. Means were separated using Fisher's LSD (ordinary pairwise *t*-test). Data effects were considered significant if $P \leq 0.05$.

3. Results

3.1. Tree phenology

In various trees of the control and Ca treatment, shoots (1-year-old wood) re-flushed during autumn (March to May 2016) (Paper 1, Fig. 5a). Regardless, leaf drop had started in most trees by mid-May 2016. A substantial amount of healthy intact leaves was still present in most trees until after 50 % leaf drop was reached in July 2016 (Paper 1, Fig. 5b). At the end of August 2016, trees reached approximately 90 % leaf drop with some leaves still firmly attached (Paper 1, Fig. 5c). This is typical of apples produced in an area with sub-optimal chilling and relatively high winter temperatures like Stellenbosch (personal communication, Dr. E. Lötze, Department of Horticultural Science, Stellenbosch University).

3.2. Root preparation

Results of the various root preparation techniques are shown in Table 1. The technique selected for this experiment, i.e. Procedure 6, proved most promising. White root tissues were adequately preserved with minimal distortion, cell shrinkage and/or collapse. Compared with the other techniques tested (Procedure 1 – 4), the current technique offered additional benefits, including fewer steps and chemicals used in tissue fixation. Although similar results were obtained with Procedure 5, the use of an alternative drying method, i.e. utilizing hexamethyldisilazane (HMDS, Sigma-Aldrich) instead of the commonly used critical point drying (CPD) (Katsen-Globa et al., 2016), was also more cost-effective, less time-consuming and required no specialized equipment or skills.

3.3. SEM-WDS analysis

No significant interaction between the main effects was found at 5 % significance level, but slight treatment effects were observed at 10 % (Fig. 4). A significant interaction was found between the main effects of time (weeks after treatment), segment and location at 5 % (Fig. 5). Because the four-way interaction was significant at 10 %, treatment effects will be highlighted where applicable.

Significant differences were found in the distribution of Ca between the apical and basal segments of the roots (Fig. 5). At three weeks after treatment, Ca concentrations in the xylem vessels of the stele ($Ca_{[stele]}$) and parenchyma cells in the cortex ($Ca_{[cortex]}$) did not differ between the apical and basal segments of the roots (Fig. 5). In both segments, Ca was equally distributed between the cortical and stellar region of the roots at that time (Fig. 5). From three to five weeks after treatment, $Ca_{[stele]}$ and $Ca_{[cortex]}$ of the apical segments of the roots increased significantly (Fig. 5). The increase in $Ca_{[cortex]}$ was most prominent in the roots of the control, while the increase in $Ca_{[stele]}$ was mostly attributed to an increase in Ca in the roots of the Ca treatment (Fig. 4). During the same uptake period, $Ca_{[stele]}$ and $Ca_{[cortex]}$ of the basal segments of the roots remained constant (Fig. 5). At five weeks after treatment, root $Ca_{[stele]}$ did not differ significantly between the apical and basal segments, while root $Ca_{[cortex]}$ was significantly higher in the apical segments compared to the basal segments (Fig. 5).

From five to seven weeks after treatment, root $Ca_{[cortex]}$ of the apical segments decreased significantly (Fig. 5). This decrease was observed in both the roots the control and Ca treatment (Fig. 4). Root $Ca_{[stele]}$ remained constant (Fig. 5) and was mostly attributed to a decrease in root $Ca_{[stele]}$ of the treatment (Fig. 4). In the basal segments, root $Ca_{[stele]}$ and $Ca_{[cortex]}$ remained constant. At seven weeks after treatment, no significant differences were found in the root $Ca_{[stele]}$ and $Ca_{[cortex]}$ between the apical and basal segments (Fig. 5).

Between seven and 11 weeks after treatment, root $Ca_{[stele]}$ and $Ca_{[cortex]}$ of the apical segments increased significantly (Fig. 5). The increase in Ca concentration was most prominent in the cortex (Fig. 5) and was attributed to an increase in Ca in both the roots of the control and Ca treatment (Fig. 4). The increase in $Ca_{[stele]}$ was primarily attributed to an increase in Ca in the roots of the control (Fig. 4). In the basal segments, root $Ca_{[stele]}$ remained constant (Fig. 5), while root $Ca_{[cortex]}$ increased significantly for both the control and Ca treatment (Fig. 4). At 11 weeks after treatment, root $Ca_{[stele]}$ was significantly higher in the apical segments compared to the basal segments, whereas root $Ca_{[cortex]}$ did not differ significantly between the apical and basal segments (Fig. 5).

From 11 to 13 weeks after treatment, $Ca_{[cortex]}$ in the apical segments remained constant (Fig. 5) in the both the roots of the control and Ca treatment (Fig. 4). Root $Ca_{[stele]}$ also remained constant (Fig. 5) due to a significant decrease in the roots of the control (Fig. 4). In the basal segments, root $Ca_{[stele]}$ and $Ca_{[cortex]}$ remained constant (Fig. 5) in both the roots of the control

and Ca treatment (Fig. 4). At 13 weeks after treatment, no significant differences were found in root $Ca_{[stele]}$ or $Ca_{[cortex]}$ between either of the segments. In both segments, root $Ca_{[cortex]}$ was significantly higher compared to root $Ca_{[stele]}$ (Fig. 5).

4. Discussion

4.1. Calcium uptake in relation to above-ground tree phenology

Scanning electron microscopy coupled with WDS was used to determine in situ concentration and distribution of Ca in apple white root tips of the control and Ca treatment over time during winter. According to Clarkson (1984), this type of analysis shows a steady-state distribution. It measures the total amount of Ca in each given location but does not permit a distinction between Ca that is bound or free and/or exchangeable. Because root Ca concentration was measured at set time intervals (weeks after treatment) and not on a continuous basis, the static view obtained from each analysis was designed to show root Ca accumulation in the two areas of interest, i.e. xylem vessels in the stele and parenchyma cells in the mid-cortex.

Calcium accumulation in the root tips was little affected by additional soil Ca applications, as no significant interaction between the main effects was found. However, it does appear as if substantial root Ca uptake and translocation to the shoots occurred, as a significant higher leaf Ca concentration was reported for the trees of the high Ca treatment (i.e. $2X_{(NLD)}$ treatment) from which the roots were harvested (Paper1). Regarding tree phenology, the shoots of various trees of the control and Ca treatment re-flushed in autumn (March to May), which advanced the 50 % leaf drop mark to mid-winter (July). This was possibly due to a combination of factors, including an ample supply of water and nutrients from fertigation as well as unusually high ambient temperatures experienced on site (Beikircher and Mayr, 2013). During this time, developing (unseasonal) and mature transpiring leaves likely provided a major sink for Ca delivery via the xylem (Shear and Faust, 1970; Clarkson, 1984; Gilliham et al., 2011). Because an abundant number of healthy intact leaves was attached to the trees during most of winter, the rate of root Ca uptake and translocation to the shoots was much faster than initially anticipated.

Biddulph (1967), Yu and Lunt (1971) and White et al. (1992) showed that the flux of Ca^{2+} to the xylem in attached roots is more rapid compared to excised roots. Within a 24-hour uptake

period, the presence of the shoot did not influence ^{45}Ca accumulation in the root tips to any significant extent, the rapidity of ^{45}Ca uptake from the external solution having possibly been regulated by the availability of ion exchange sites in the cortical apoplast. Calcium movement through the apoplast is not free, but dependent on the structure of the extracellular matrix, particularly, on the amount of non-diffusible anions located in the cell walls (Haynes, 1980; McLaughlin and Wimmer, 1999; Sattelmacher, 2001; Gilliham et al., 2011). Biddulph (1967) showed that the ion exchange sites in the cortical apoplast of intact bean roots were saturated with ^{45}Ca within 1.25 hours of uptake. Presumably, when the flow of transpiration carried more ^{45}Ca ions to the surface than could be accommodated for on the sites, Ca^{2+} movement across the cortex proceeded via exchange with other Ca^{2+} ions held on the exchange sites by adsorptive forces. Depending on the solution concentration, the number and loading status of the exchange sites in the xylem column, and the intensity of transpiration (Biddulph et al., 1961; Bell and Biddulph, 1963; Ferguson and Bollard, 1976; Van de Geijn and Petit, 1979), after the release of Ca^{2+} ions to the xylem, the increase in xylem Ca concentration is momentary. According to Yu and Lunt (1971), the Ca concentration in the xylem column of maize roots decreased rapidly within the first hour of uptake as Ca^{2+} ions proceeded to move upwards in the xylem sap. In agreement, Biddulph (1967) found an increasing amount of ^{45}Ca inside the stele of bean roots with an increasing distance from the root tip within 1.25 hours of uptake. Our results corroborate these previous findings, since, in all the root segments analysed, no significant differences in root Ca concentration between the control and Ca treatment were found at 5 % significance level. The first batch of roots was harvested three weeks after $Ca(NO_3)_2$ was applied to the soil, followed by bi-weekly to monthly harvests. The time between root harvests was possibly too far apart to observe treatment effects in the roots at each time-point. However, at a 10 % significance level, slight treatment effects between time intervals enabled us to characterize the uptake and radial movement of Ca^{2+} across the length of apple white root tips with some certainty, based on its distribution.

4.2. Calcium distribution along the length of apple root tips

At five weeks after treatment (21 June 2016), the higher root $Ca_{[cortex]}$ of the apical segments was attributed to an increase in Ca concentration in the roots of the control and not to an increase in Ca concentration in the roots of the Ca treatment, as the latter increase was not significant. This suggests that the Ca concentration of the nutrient solution (fertigation) was probably sufficient to saturate the ion exchange sites along the cortical apoplast of the roots of

the control, while the concentration of the Ca treatment was high enough to allow translocation of additional Ca^{2+} ions across the endodermis to the stele at a rate that matched transpirational demand. The latter was indicated by the significant increase in root $Ca_{[stele]}$ in the apical segments of the Ca treatment at that time. Although the distribution of Ca in the cortex between the apical and basal root segments was similar for the control and Ca treatment at that time, the Ca concentration of the basal root segments was significantly lower compared to the apical root segments. Since there were no significant differences in Ca concentration between the cortex and stele of the basal segments at that time, these observations suggest that Ca^{2+} translocation to the shoot proceeded mainly via the apoplast, based on the findings of Cholewa and Peterson (2004). As the basal region of the root most likely contained functional xylem vessels in the stele (Riedhart and Guard, 1957; Mackenzie, 1979), Ca entry via the apoplast possibly occurred through impartially developed Casparian bands in the endodermis or through momentary discontinuities in the Casparian bands associated with the initiation of lateral root primordia in the pericycle (Ferguson and Clarkson, 1975; White and Broadley, 2003). This, however, could not be determined from the SEM-WDS images (data not shown). Moreover, at that time, root $Ca_{[stele]}$ of the Ca treatment was significantly lower in the basal compared to the apical segments, whereas in the control, root $Ca_{[stele]}$ did not differ significantly between the apical and basal segments. If the ion exchange sites in the xylem column was saturated at that point, it seems probable that the Ca^{2+} ions in the stele of the Ca treatment was already transported upwards in the xylem sap at a rate that matched transpirational demand.

At three and seven weeks after treatment (10 June 2016, 7 July 2016), the distribution of Ca in the cortex and stele was similar in both root segments of the control and Ca treatment, suggesting that Ca^{2+} translocation to the shoot proceeded mainly via the apoplast at a rate that matched transpirational demand. If endodermal Casparian bands were present, Ca^{2+} translocation to the shoot would have had to proceed via the symplast. However, if Ca^{2+} movement across the endodermis proceeded solely via the symplast, the amount of Ca in the stele compared to the cortex should have been substantially reduced (Cholewa and Peterson, 2004). In support, in mycorrhizal spruce roots, Kuhn et al. (2000) showed that the rate of Ca^{2+} entry into the stele was two orders of magnitude slower than entry into the cortex due to the diffusion barrier properties of the endodermis. Although membrane embedded Ca^{2+}-permeable channels are capable of catalysing substantial Ca^{2+} influx across the plasma membrane of root endodermal cells when active (Hayter and Peterson, 2004; Wang et al., 2006; Yang et al., 2011), at times of high shoot demand, the density and capacity of plasma membrane Ca^{2+}-

ATPase transporters may not be sufficient to discharge such large quantities of Ca^{2+} into the stellar apoplast (White, 1998, 2001; White and Broadley, 2003). Thus, since $Ca_{[cortex]}$ and $Ca_{[stele]}$ did not differ significantly between either of the root segments of the control and Ca treatment, leaf transpiration could have accounted for a high rate of Ca^{2+} translocation to the shoot via the apoplast if the ion exchange sites along the cortical and stellar apoplast of the root tips of both treatments were saturated at those times, and if endodermal Casparian bands were absent or disrupted along the entire length of the root tips at those times.

At 11 and 13 weeks after treatment (10, 25 August 2016), there was a clear separation in Ca distribution between the cortex and the stele in both root segments of the control and Ca treatment. Root $Ca_{[cortex]}$ was significantly higher compared to root $Ca_{[stele]}$ in both segments. These observations differ substantially from those prior to 11 weeks after treatment, when the trees were still mostly in leaf. The marked increase in Ca concentration in the root tips harvested in August was probably not due an increase in the rate of Ca uptake by the roots, as the trees were mostly leafless, but rather due to accumulation, as the demand for Ca by the shoots would have been relatively low towards the end of winter. In peach trees, Richards and Rowe (1977) found an increase in nutrient absorption per unit root length with an increase in the size of the shoot, and concluded that the rate of root nutrient uptake is strongly related to shoot demand. In maize plants, Engels (1999) studied the effect of growth-related nutrient demand on the rate of Ca^{2+} translocation to the shoot. In one group of plants, shoot demand was diminished by a reduction in shoot meristem temperature, and in another, by decapitation. Root growth was not affected by either treatment. As both treatments led to a marked reduction in Ca^{2+} translocation from the roots to the shoot, it was suggested that a decrease in Ca^{2+} flux at low shoot demand is not only related to a decrease in transpiration-driven water flux but is also due to a suppression in root transport capacity for Ca^{2+}, the latter possibly brought on by a decrease in Ca^{2+}-ATPase activity in the endodermis. In agreement, White and Broadley (2003) reported that plants can regulate the expression and activity of plasma membrane protein transporters in their root endodermal cells to adapt the rate of Ca^{2+} translocation to the shoot according to metabolic demand.

It may be possible that the differential patterns of Ca distribution in the roots harvested before and after 50 % leaf drop was related to differences in their anatomy associated with age (Mackenzie, 1979; Clarkson, 1984; White, 2001; Wells and Eissenstat, 2003; Volder et al., 2005) or root growth rate (Wilcox, 1962; Ferguson and Clarkson, 1975; Waisel and Eshel,

2002; Enstone et al., 2003; Ma and Peterson, 2003). Since we consistently selected only white pioneer roots for this experiment, differences in their anatomy was not likely related to age, as McCrady and Comerford (1998) reported that the white colour of young roots is a reliable indicator of their primary developmental state. Thus, based on previous observations of the effect of root growth rate on root endodermal development (Wilcox, 1962; Waisel and Eshel, 2002; Enstone et al., 2003), our results suggest that roots harvested before July (prior to the 50 % leaf drop mark) had relatively high elongation rates, which were possibly sustained by a steady supply of construction material and assimilates from the healthy intact leaves that were still attached to the trees, while roots harvested after July (past the 50 % leaf drop mark) had relatively low elongation rates. However, neither root growth rate nor photosynthesis were quantified. In contrast with most roots harvested before July, the significantly higher $Ca_{[cortex]}$ of the roots harvested after July suggests Ca^{2+} movement across the endodermis to the stele proceeded predominately via the symplast along the length of relatively slow growing roots. Based on the findings of Engels (1999) and White and Broadley (2003), this would have allowed the roots to selectively deliver Ca^{2+} to the xylem at a rate consistent with the requirements of the shoot towards the end of winter.

Despite a lack of direct evidence on root endodermal development, the results of the SEM-WDS analyses indicated rapid uptake of soil-applied Ca along the entire length of apple white root tips during winter and allowed us to suggest that both apoplastic and symplastic pathways contribute to Ca^{2+} delivery to apple shoots via the xylem during winter. However, the importance of applying multiple methodologies toward a broader understanding of soil Ca uptake and translocation from apple root tips during winter is clear. We propose a combination of SEM-WDS/EDS analysis, histochemical staining of lignin and suberin for light microscopy investigations and/or fluorescence microscopy with or without staining, as described by Tuladhar and Nii (2014), for concurrent determinations of root development stages. Scanning electron microscopy coupled with EDS is useful in establishing nutrient element distribution maps on the surfaces of plant tissues (Cocozza et al., 2008; Hunsche and Noga, 2008), which could also aid future investigations.

Additional measurements, including leaf transpiration, photosynthesis and root growth rate also serve as points for improvement for future studies. When using minirhizotrons, one option for measuring root growth rate is to use digital imaging combined with automated analysis through computer software programs, e.g. WinRHIZO, to record root length in daily/weekly

increments over time (Judd et al., 2015). Another option is a method described by Bouma et al. (2001) and Resendes et al. (2008) utilizing buried root observation boxes (i.e. rhizo-boxes) containing a clear acetate window and tracing individual roots with coloured markers directly on the window from the time of their initiation. Daily length increases can be measured with a ruler. An advantage of this method is that roots of known ages can be identified and individually sampled for analysis by cutting through the acetate window. Clear containers or plastic pots out of which a window can be cut and replaced with an acetate sheet can also be used (Judd et al., 2015).

5. Conclusion

From this experiment and other work presented in Paper 1, it was concluded that the rate of soil-applied Ca uptake by white root tips of young, potted, non-bearing 'Golden Delicious'/M7 apple trees during winter in the Western Cape was rapid, possibly because the trees were in leaf for an extended period. Following additional soil Ca applications in autumn, estimates of root Ca uptake over time were affected by the root harvesting schedule. Starting at three weeks after treatment, bi-weekly to monthly determinations proved too far apart to reflect treatment effects at each time-point. To perform SEM-WDS analyses on root tips on a continuous basis (less than 24 hours apart) would not only be costly but would require an immense number of roots to be harvested from trees. Thus, SEM-WDS analysis may be more useful for the study of soil Ca uptake by white root tips over a brief period.

Calcium uptake and translocation took place along the entire length of the root tip (at least up to 20 mm from the apex). Since root growth rate is a determining factor for the distance from the root apex at which endodermal Casparian band development commences, the pathway of Ca translocation to the shoot during winter seemed to depend on root growth rate. Furthermore, the rate of Ca translocation to the shoot seemed to be governed by both transpirational and growth-related demand. The results of this pilot study suggest that when trees were still mostly in leaf (up to July), Ca translocation to the shoot proceeded via the apoplastic pathway in faster growing roots at a rate that matched transpirational demand. By contrast, when leaf drop surpassed the 50 %-mark, Ca translocation to the shoot possibly proceeded via the symplastic pathway in slower growing roots at a rate that matched shoot demand. At low shoot demand towards the end of winter, apple roots may regulate Ca translocation to the shoot by suppressing

endodermal membrane transporter activity, based on previous findings. These measurements fell outside the scope of this experiment and deserves further investigation.

Calcium distribution patterns along the length of apple white root tips during winter following late-autumn soil $Ca(NO_3)_2$ applications during a period of active white root growth could be established using SEM-WDS. Yet, results only allowed us to distinguish between the apoplastic and symplastic pathways of Ca translocation to the shoot with some certainty, as concurrent digital images did not provide the required information on internal root development stages during winter. A combination of SEM-WDS/EDS analysis and histochemical staining for light microscopy and/or fluorescence microscopy investigations is therefore advised.

Acknowledgements

Yara Africa Fertilizer (Pty). Ltd. funded this experiment. We would also like to thank the following people: Dr. S. Turketti for her insightful suggestions in this experimental study, Mrs. M. Waldron at the Electron Microscope Unit (University of Cape Town) for her assistance with root preparation, Mrs. M. Frazenburg at the CAF laboratory (Stellenbosch University) for her assistance with SEM analysis, and Prof. M. Kidd for his assistance with statistical analysis.

References

Atkinson, D., 1983. The growth, activity and distribution of the fruit tree root system. Plant Soil 71, 23–35.

Atkinson, D., Wilson, S.A., 1979. The root-soil interface and its significance for fruit tree roots of different ages, in: Harley, J.L., Russel, R.S. (Eds.), The Soil-Root Interface. Academic Press, London, pp. 259–271.

Atkinson, D., Wilson, S.A., 1980. The growth and distribution of fruit tree roots: Some consequences for nutrient uptake, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 137–150.

Bagniewska-Zadworna, A., Byczyk, J., Eissenstat, D.M., Oleksyn, J., Zadworny, M., 2012. Avoiding transport bottlenecks in an expanding root system: Xylem vessel development in fibrous and pioneer roots under field conditions. Am. J. Bot. 99, 1417–1426.

Balasubramaniyam, A., Harvey, P.J., 2014. Scanning electron microscopic investigations of root structural modifications arising from growth in crude oil-contaminated sand. Environ. Sci. Pollut. Res. 21, 12651–12661.

Baldi, E., Wells, C.E., Marangoni, B., 2010. Nitrogen absorption and respiration in white and brown peach roots. J. Plant Nutr. 33, 461–469.

Beikircher, B., Mayr, S., 2013. Winter peridermal conductance of apple trees: Lammas shoots and spring shoots compared. Trees 27, 707–715.

Bell, C.W., Biddulph, O., 1963. Translocation of calcium. Exchange versus mass flow. Plant Physiol. 38, 610–614.

Biddulph, O., Nakayama, F.S., Cory, R., 1961. Transpiration stream and ascension of calcium. Plant Physiol. 36, 429–436.

Biddulph, S.F., 1967. A microautoradiographic study of ^{45}Ca and ^{35}S distribution in the intact bean root. Planta 74, 350–367.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Chino, M., 1979. Calcium localization with plant roots by electronprobe x-ray microanalysis.

Commun. Soil Sci. Plant Anal. 10, 443–457.

Chino, M., Baba, A., 1976. Electron microprobe analysis of zinc and other elements within and around rice root grown in flooded soil. Soil Sci. Plant Nutr. 22, 445–457.

Chino, M., Hidaka, H., 1977. Direct observation and x-ray microanalysis of frozen specimens of plant root in soil. Soil Sci. Plant Nutr. 23, 195–200.

Cholewa, E., Peterson, C.A., 2004. Evidence for symplastic involvement in the radial movement of calcium in onion roots. Plant Physiol. 134, 1793–1802.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

Clode, P.L., 2015. A method for preparing difficult plant tissues for light and electron microscopy. Microsc. Microanal. 21, 902–909.

Cocozza, C., Minnocci, A., Tognetti, R., Iori, V., Zacchini, M., Scarascia Mugnozza, G., 2008. Distribution and concentration of cadmium in root tissue of *Populus alba* determined by scanning electron microscopy and energy-dispersive x-ray microanalysis. IForest 1, 96–103.

Comas, L.H., Bauerle, T.L., Eissenstat, D.M., 2010. Biological and environmental factors controlling root dynamics and function: Effects of root ageing and soil moisture. Aust. J. Grape Wine Res. 16, 131–137.

Comerford, N.B., Smethurst, P.J., Escamilla, J.A., 1994. Nutrient uptake by woody root systems. New Zeal. J. For. Sci. 24, 195–212.

Crider, F.J., 1933. Selective absorption of ions not confined to young rootlets. Science 78, 169.

Damunupola, J.W., Ratnayake, K., Joyce, D.C., Irving, D.E., 2011. Characterisation of xylem conduits and their possible role in limiting the vase life of cut *Acacia holosericea* (*Mimosaceae*) foliage stems. Funct. Plant Biol. 38, 614–623.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hort. Rev. 40, 107–146.

Eissenstat, D.M., Achor, D.S., 1999. Anatomical characteristics of roots of citrus rootstocks that vary in specific root length. New Phytol. 141, 309–321.

Engels, C., 1999. Regulation of xylem transport of calcium from roots to shoot of maize by growth-related demand. J. Plant Nutr. Soil Sci. 162, 287–294.

Enstone, D.E., Peterson, C.A., Ma, F., 2003. Root endodermis and exodermis: Structure, function, and responses to the environment. J. Plant Growth Regul. 21, 335–351.

Escamilla, J.A., Comerford, N.B., 2000. Phosphorus and potassium uptake by woody roots of twelve-year-old slash pine trees. For. Ecol. Manage. 129, 153–166.

Ferguson, I.B., 1980. The uptake and transport of calcium in the fruit tree, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 183–192.

Ferguson, I.B., Bollard, E.G., 1976. The movement of calcium in woody stems. Ann. Bot. 40, 1057–1065.

Ferguson, I.B., Clarkson, D.T., 1975. Ion transport and endodermal suberization in the roots of *Zea mays*. New Phytol. 75, 69–79.

Ferguson, I.B., Clarkson, D.T., 1976. Simultaneous uptake and translocation of magnesium and calcium in barley (*Hordeum vulgare* L.) roots. Planta 128, 267–269.

Geldner, N., 2013. The endodermis. Annu. Rev. Plant Biol. 64, 531–558.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Gu, J., Wei, X., Wang, J., Dong, X., Wang, Z., 2015. Marked differences in standing biomass, length density, anatomy and physiological activity between white and brown roots in *Fraxinus mandshurica* Rupr. plantation. Plant Soil 392, 267–277.

Guo, D., Xia, M., Wei, X., Chang, W., Liu, Y., Wang, Z., 2008. Anatomical traits associated with absorption and mycorrhizal colonization are linked to root branch order in twenty-three Chinese temperate tree species. New Phytol. 180, 673–683.

Harrison-Murray, R.S., Clarkson, D.T., 1973. Relationships between structural development and the absorption of ions by the root system of *Cucurbita pepo*. Planta 114, 1–16.

Hawkins, B.J., Robbins, S., Porter, R.B., 2014. Nitrogen uptake over entire root systems of tree seedlings. Tree Physiol. 34, 334–342.

Haynes, R.J., 1980. Ion exchange properties of roots and ionic interactions within the root

apoplasm: Their role in ion accumulation by plants. Bot. Rev. 46, 75–99.

Hayter, M.L., Peterson, C.A., 2004. Can Ca^{2+} fluxes to the root xylem be sustained by Ca^{2+}-ATPases in exodermal and endodermal plasma membranes? Plant Physiol. 136, 4318–4325.

Head, G.C., 1966. Estimating seasonal changes in the quantity of white suberized root on fruit trees. J. Hort. Sci. 41, 197–206.

Hishi, T., 2007. Heterogeneity of individual roots within the fine root architecture: Causal links between physiological and ecosystem functions. J. For. Res. 12, 126–133.

Huang, J., Cai, Z., Wen, S., Guo, P., Ye, X., Lin, G., Chen, L., 2014. Effects of boron toxicity on root and leaf anatomy in two citrus species differing in boron tolerance. Trees 28, 1653–1666.

Hunsche, M., Noga, G., 2008. Applicability of the energy dispersive x-ray microanalysis for quantification of irregular calcium deposits on fruit and leaf cuticles. J. Microsc. 232, 453–462.

Joshi, R., Shukla, A., Mani, S.C., Kumar, P., 2010. Hypoxia induced non-apoptotic cellular changes during aerenchyma formation in rice (*Oryza sativa* L.) roots. Physiol. Mol. Biol. Plants 16, 99–106.

Judd, L., Jackson, B., Fonteno, W., 2015. Advancements in root growth measurement technologies and observation capabilities for container-grown plants. Plants 4, 369–392.

Kamula, S.A., Peterson, C.A., Mayfield, C.I., 1994. The plasmalemma surface area exposed to the soil solution is markedly reduced by maturation of the exodermis and death of the epidermis in onion roots. Plant Cell Environ. 17, 1183–1193.

Katsen-Globa, A., Puetz, N., Gepp, M.M., Neubauer, J.C., Zimmermann, H., 2016. Study of SEM preparation artefacts with correlative microscopy: Cell shrinkage of adherent cells by HMDS-drying. Scanning 38, 625–633.

Kuhn, A.J., Schröder, W.H., Bauch, J., 2000. The kinetics of calcium and magnesium entry into mycorrhizal spruce roots. Planta 210, 488–496.

Kumar, P., Hallgren, S.W., Enstone, D.E., Peterson, C.A., 2007. Root anatomy of *Pinus taeda* L.: Seasonal and environmental effects on development in seedlings. Trees 21, 693–706.

Ma, F., Peterson, C.A., 2003. Current insights into the development, structure and chemistry

of the endodermis and exodermis of roots. Can. J. Bot. 81, 405–421.

Mackenzie, K.A.D., 1979. The development of the endodermis and phi layer of apple roots. Protoplasma 100, 21–32.

Majdi, H., Damm, E., Nylund, J.-E., 2001. Longevity of mycorrhizal roots depends on branching order and nutrient availability. New Phytol. 150, 195–202.

McCormack, M.L., Dickie, I.A., Eissenstat, D.M., Fahey, T.J., Fernandez, C.W., Guo, D., Helmisaari, H.-S., Hobbie, E.A., Iversen, C.M., Jackson, R.B., Leppalammi-Kujansuu, J., Norby, R.J., Phillips, R.P., Pregitzer, K.S., Pritchard, S.G., Rewald, B., Zadworny, M., 2015. Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes. New Phytol. 207, 505–518.

McCormack, M.L., Guo, D., Iversen, C.M., Chen, W., Eissenstat, D.M., Fernandez, C.W., Li, L., Ma, C., Ma, Z., Poorter, H., Reich, P.B., Zadworny, M., Zanne, A., 2017. Building a better foundation: Improving root-trait measurements to understand and model plant and ecosystem processes. New Phytol. 215, 27–37.

McCrady, R.L., Comerford, N.B., 1998. Morphological and anatomical relationships of loblolly pine fine roots. Trees 12, 431–437.

Mckenzie, B.E., Peterson, C.A., 1995a. Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 1. Anatomy and permeability of the white and tannin zones. Bot. Acta 108, 127–137.

Mckenzie, B.E., Peterson, C.A., 1995b. Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 2. Anatomy and permeability of the cork zone. Bot. Acta 108, 138–143.

McLaughlin, S.B., Wimmer, R., 1999. Calcium physiology and terrestrial ecosystem processes. New Phytol. 142, 373–417.

Meyer, C.J., Peterson, C.A., 2013. Structure and function of three suberized cell layers: Epidermis, exodermis and endodermis, in: Eshel, A., Beeckman, T. (Eds.), Plant Roots: The Hidden Half. CRC Press, Boca Raton-London-New York, p. 5.1-5.20.

Nightingale, G.T., 1935. Effects of temperature on growth, anatomy, and metabolism of apple and peach roots. Bot. Gaz. 96, 581–639.

Pathan, A.K., Bond, J., Gaskin, R.E., 2008. Sample preparation for scanning electron

microscopy of plant surfaces - horses for courses. Micron 39, 1049–1061.

Peterson, C.A., Emanuel, M.E., Weerdenburg, C.A., 1981. The permeability of phi thickenings in apple (*Pyrus malus*) and geranium (*Pelargonium hortorum*) roots to an apoplastic fluorescent dye tracer. Can. J. Bot. 59, 1107–1110.

Peterson, C.A., Enstone, D.E., 1996. Functions of passage cells in the endodermis and exodermis of roots. Physiol. Plant. 97, 592–598.

Peterson, C.A., Enstone, D.E., Taylor, J.H., 1999. Pine root structure and its potential significance for root function. Plant Soil 217, 205–213.

Polverigiani, S., Kelderer, M., Neri, D., 2014. Growth of 'M9' apple root in five Central Europe replanted soils. Plant Root 8, 55–63.

Polverigiani, S., McCormack, M.L., Mueller, C.W., Eissenstat, D.M., 2011. Growth and physiology of olive pioneer and fibrous roots exposed to soil moisture deficits. Tree Physiol. 31, 1228–1237.

Resendes, M.L., Bryla, D.R., Eissenstat, D.M., 2008. Early events in the life of apple roots: variation in root growth rate is linked to mycorrhizal and nonmycorrhizal fungal colonization. Plant Soil 313, 175–186.

Rewald, B., Rechenmacher, A., Godbold, D.L., 2014. It's complicated: Intraroot system variability of respiration and morphological traits in four deciduous tree species. Plant Physiol. 166, 736–745.

Richards, D., Considine, J.A., 1981. Suberization and browning of grapevine roots, in: Brouwer, R., Gasparikova, O., Kolek, J., Loughman, B.C. (Eds.), Structure and Function of Plant Roots. Dr. W. Junk, London, pp. 111–115.

Richards, D., Rowe, R.N., 1977. Root-shoot interactions in peach: The function of the root. Ann. Bot. 41, 1211–1216.

Riedhart, A.W., Guard, A.T., 1957. On the anatomy of the roots of apple seedlings. Bot. Gaz. 118, 191–194.

Ryan, P.R., Newman, I.A., Shields, B., 1990. Ion fluxes in corn roots measured by microelectrodes with ion-specific liquid membranes. J. Memb. Sci. 53, 59–69.

Sattelmacher, B., 2001. The apoplast and its significance for plant mineral nutrition. New Phytol. 149, 167–192.

Schreiber, L., Hartmann, K., Skrabs, M., Zeier, J., 1999. Apoplastic barriers in roots: Chemical composition of endodermal and hypodermal cell walls. J. Exp. Bot. 50, 1267–1280.

Shear, C.B., Faust, M., 1970. Calcium transport in apple trees. Plant Physiol. 45, 670–674.

Song, Y., Ye, L., Nii, N., 2011. Effects of soil water availability on development of suberin lamellae in the endodermis and exodermis and on cortical cell wall thickening in red bayberry (*Myrica rubra* Sieb. et Zucc.) tree roots. Sci. Hort. 129, 554–560.

Storey, R., Leigh, R.A., 2004. Processes modulating calcium distribution in citrus leaves. An investigation using x-ray microanalysis with strontium as a tracer. Plant Physiol. 136, 3838–3848.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Tuladhar, A., Nii, N., 2014. An anatomical study of developmental changes in maturing root tissues of pomegranate (*Punica granatum* L.) and formation of a unique type of periderm. Plant Root 8, 33–41.

Ubero-Pascal, N., Fortuño, J.M., De Los Ángeles Puig, M., 2005. New application of air-drying techniques for studying *Ephemeroptera* and *Plecoptera* eggs by scanning electron microscopy. Microsc. Res. Tech. 68, 264–271.

Van de Geijn, S.C., Petit, C.M., 1979. Transport of divalent cations. Cation exchange capacity of intact xylem vessels. Plant Physiol. 64, 954–958.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Volder, A., Smart, D.R., Bloom, A.J., Eissenstat, D.M., 2005. Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: Implications for root efficiency and competitive effectiveness. New Phytol. 165, 493–501.

Waisel, Y., Eshel, A., 2002. Functional diversity of various constituents of a single root sytem, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel

Dekker, Inc., New York, NY, USA, pp. 157–174.

Wang, H., Inukai, Y., Yamauchi, A., 2006. Root development and nutrient uptake. Crit. Rev. Plant Sci. 25, 279–301.

Weerdenburg, C.A., Peterson, C.A., 1983. Structural changes in phi thickenings during primary and secondary growth in roots. 1. Apple (*Pyrus malus*) *Rosaceae*. Can. J. Bot. 61, 2570–2576.

Wells, C.E., Eissenstat, D.M., 2003. Beyond the roots of young seedlings: The influence of age and order on fine root physiology. Plant Growth Regul. 21, 324–334.

White, P.J., 1998. Calcium channels in the plasma membrane of root cells. Ann. Bot. 81, 173–183.

White, P.J., 2001. The pathways of calcium movement to the xylem. J. Exp. Bot. 52, 891–899.

White, P.J., Banfield, J., Diaz, M., 1992. Unidirectional Ca^{2+} fluxes in roots of rye (*Secale cereale* L). A comparison of excised roots with roots of intact plants. J. Exp. Bot. 43, 1061–1074.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilcox, H., 1962. Growth studies of the root of incense cedar, *Libocedrus decurrens*. I. The origin and development of primary tissues. Am. J. Bot. 49, 221–236.

Yang, H., Jie, Y., Shao, X., 2011. Regulation of calcium uptake and translocation in plants. Acta Hort. 903, 1011–1016.

Yu, G.H., Lunt, O.R., 1971. Radial transport of calcium in corn roots (No. UCLA-12-811). California University, Los Angeles, CA, USA.

Zadworny, M., Eissenstat, D.M., 2011. Contrasting the morphology, anatomy and fungal colonization of new pioneer and fibrous roots. New Phytol. 190, 213–221.

Table 1. The outcome of various tissue preparation procedures for subsequent SEM-WDS analysis involving fixation/post-fixation with different combinations of chemicals and two different drying techniques.

Procedure	Steps +	References ++	Observations
1	Fixation: 2.5 % GA (*v*/*v*) for 24 h at 4 °C Rinse: PBS Post-fixation: 1 % OsO_4 (*w*/*v*) for 3 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: CPD (1-1.5 h)	(Cocozza et al., 2008) (Balasubramaniyam and Harvey, 2014)	Shrinkage/collapse of the epidermis, outer cortex and stele. Cell structure in the mid/inner cortex well preserved (Annexure 2, Fig. 1A).
2	Fixation: 2.5 % GA (*v*/*v*) for 24 h at 4 °C Rinse: PBS Post-fixation: 1 % OsO_4 (*w*/*v*) for 3 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: 100 % HMDS (air-dried for 25-30 min)	(Ubero-Pascal et al., 2005) (Cocozza et al., 2008) (Damunupola et al., 2011) (Balasubramaniyam and Harvey, 2014) (Katsen-Globa et al., 2016)	Epidermis, outer cortex and stele better preserved compared to CPD (Procedure 1). Slight shrinkage/distortion of cells in mid-cortex (Annexure 2, Fig. 1B).
3	Fixation: Modified Karnovsky's fixative for 24 h at 4 °C Rinse: PBS Post-fixation: 1 % OsO_4 for 3 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: CPD (1-1.5 h)	(Joshi et al., 2010) (Huang et al., 2014)	Epidermis, outer cortex and stele well preserved. Shrinkage/distortion of cells in selected areas of mid-cortex (Annexure 2, Fig. 1C).

Procedure	Steps +	References ++	Observations
4	Fixation: Modified Karnovsky's fixative for 24 h at 4 °C Rinse: PBS Post-fixation: 1% OsO_4 for 3 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: 100 % HMDS (air-dried for 25-30 min)	(Ubero-Pascal et al., 2005) (Joshi et al., 2010) (Damunupola et al., 2011) (Huang et al., 2014) (Katsen-Globa et al., 2016)	Shrinkage/collapse of cells in epidermis and cortex. Stele adequately preserved (Annexure 2, Fig. 1D). CPD provided better tissue preservation compared to HMDS drying.
5	Fixation: 2.5 % GA (*v*/*v*) for 24 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: CPD (1-1.5 h)	(Cocozza et al., 2008)	Slight tear (possibly caused during sectioning/handling) visible in the cortex. Tissues well preserved except for slight shrinkage of cells in mid-cortex (Annexure 2, Fig. 1E).
6	Fixation: 2.5 % GA (*v*/*v*) for 24 h at 4 °C Rinse: PBS (× 3) Dehydration: ethanol-water solutions (20, 30, 40, 60, 80, 90, 95, 100 % for 10 min each) Drying: 100 % HMDS (air-dried for 25-30 min)	(Ubero-Pascal et al., 2005) (Cocozza et al., 2008) (Damunupola et al., 2011) (Katsen-Globa et al., 2016)	No visible difference in tissue preservation compared to CPD (Procedure 5) (Annexure 2, Fig. 1F). HMDS drying was less time-consuming, required less skill and was more cost-effective. Preservation of cell structure was not negatively affected by the exclusion of post-fixation with OsO_4 (Procedure 2).

+ GA – Glutaraldehyde (0.1 M phosphate buffer, pH 7.2); PBS – Phosphate buffer solution (pH 7.2); OsO_4 – Osmium tetroxide (in distilled water); Modified Karnovsky's fixative – 3 % Glutaraldehyde + 2 % Paraformaldehyde (0.1 M Phosphate buffer solution, pH 7.2); CPD – critical point drying with E3000 Critical Point Drier (Quorum Technologies Inc., UK); HMDS – hexamethyldisilazane (Sigma-Aldrich).

++ Preparation procedures sourced from literature were modified to accommodate for the availability of chemicals and equipment.

Fig. 1. Image of a white pioneer root (right) harvested by hand from the bigger root mass of a one-year-old, potted, non-bearing 'Golden Delicious'/M7 apple tree before it was cut at approximately 30 mm from the apex.

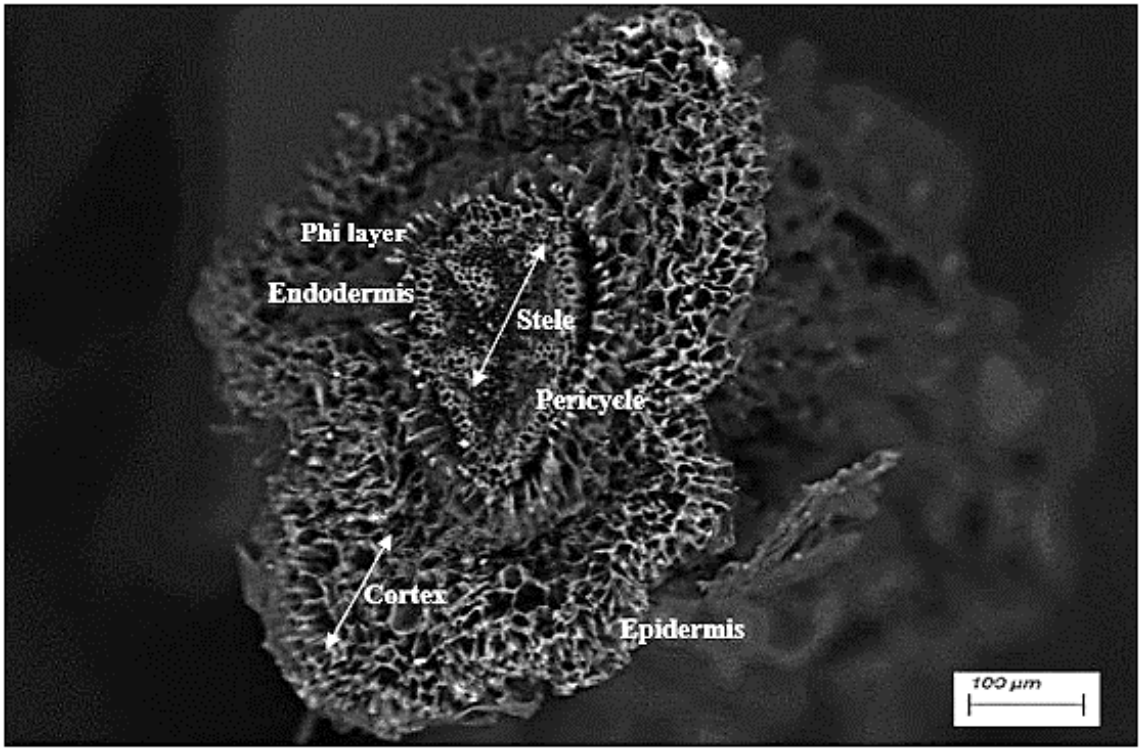


Fig. 2. Scanning electron micrograph of the transverse surface, 5 mm from the apex (apical segment), of a white root of a one-year-old, potted, 'Golden Delicious'/M7 apple tree. The different tissues in the root tip are indicated (Mackenzie, 1979; Peterson et al., 1981; Weerdenburg and Peterson, 1983).

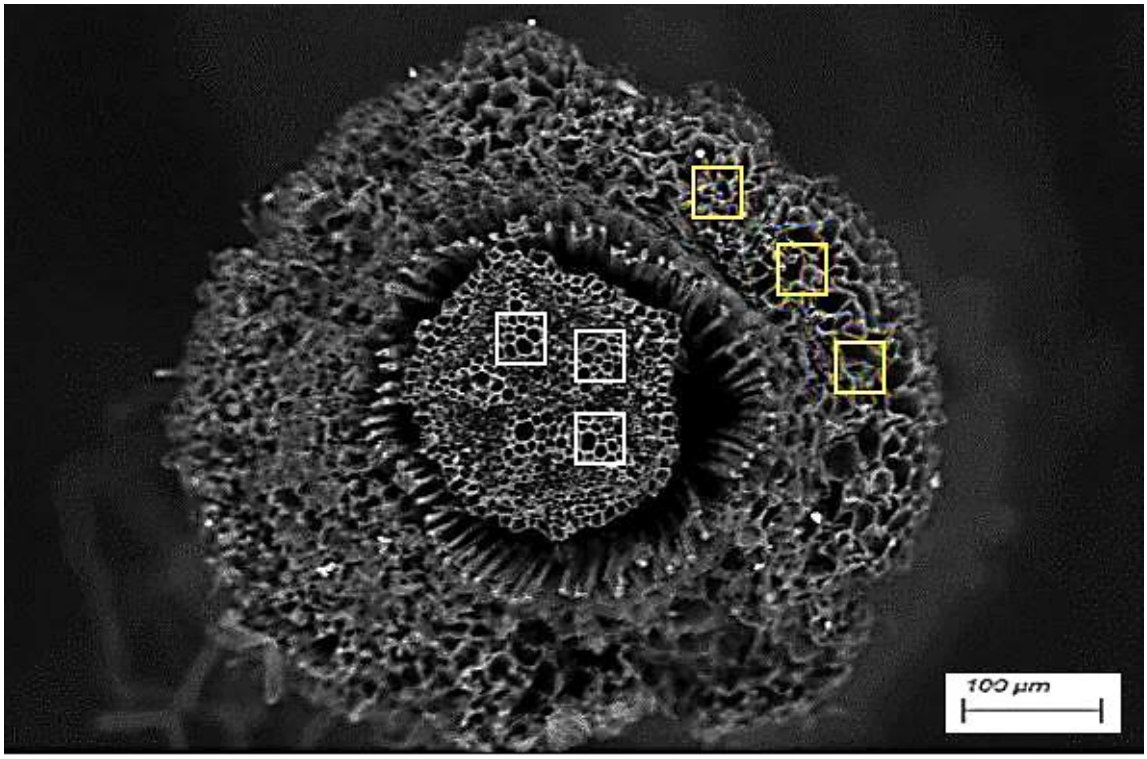
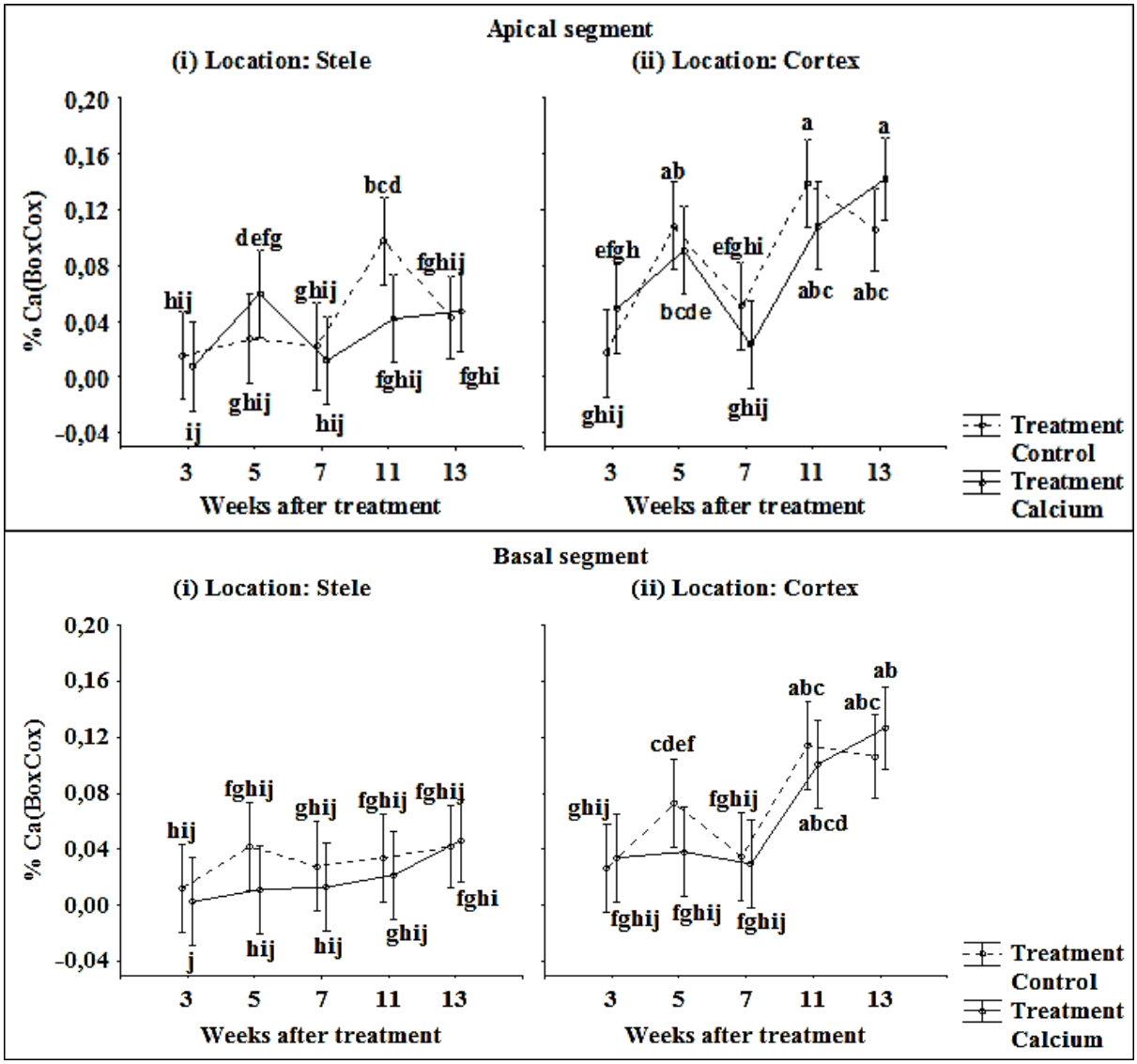
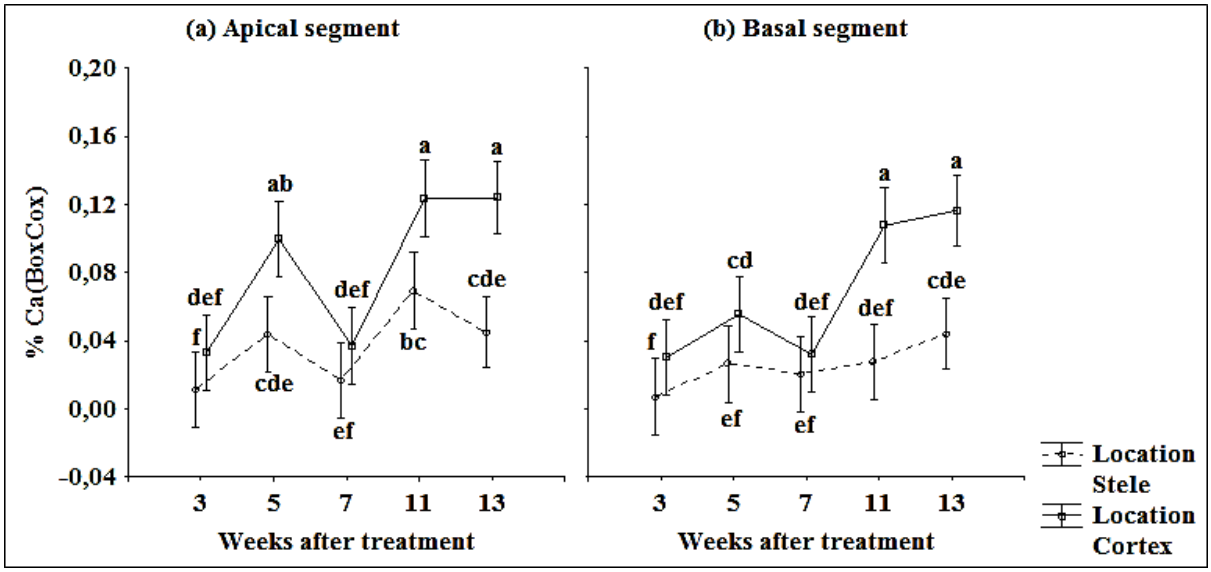


Fig. 3. Scanning electron micrograph of a cross-section cut at 20 mm from the apex (basal segment) of a white root of a one-year-old, potted, 'Golden Delicious'/M7 apple tree. The blocks give an estimated depiction of the three points where spot analyses were performed using SEM-WDS, to determine the Ca concentration in the two areas of interest: white blocks – xylem vessels in the stele; yellow blocks – parenchyma cells in the mid-cortex.



ANOVA	F-value	Pr > F
Treatment × Weeks after treatment × Segment	1.99	0.1341
Treatment × Weeks after treatment × Location	0.85	0.5090
Treatment × Segment × Location	0.03	0.8552
Weeks after treatment × Segment × Location	**2.73**	**0.0292**
Treatment × Weeks after treatment × Segment × Location	2.10	0.0802

Fig. 4. Interaction effects of treatment, time (weeks after treatment), segment (apical 5 mm from root tip, and basal 20 mm from root tip) and location (stele and cortex) on the Ca concentration (weight %) in white root tips of one-year-old, potted, 'Golden Delicious'/M7 apple trees during winter. Treatments included a calcium treatment comprising high $Ca(NO_3)_2$ soil applications, and a control that received no additional soil applications. Ca concentration was determined using SEM-WDS. BoxCox transformed data show the mean ± SE. *t*-test comparison between treatments is also reported ($P \leq 0.10$).



ANOVA	F-value	Pr > F
Weeks after treatment × Segment	2.64	0.0645
Weeks after treatment × Location	4.75	0.0074
Segment × Location	0.36	0.5476
Weeks after treatment × Segment × Location	**2.73**	**0.0292**

Fig. 5. Interaction effects of time (weeks after treatment), segment (apical 5 mm from root tip, and basal 20 mm from root tip) and location (stele and cortex) on the Ca concentration (weight %) in white root tips of one-year-old, potted, 'Golden Delicious'/M7 apple trees during winter. Ca concentration was determined using SEM-WDS. BoxCox transformed data show the mean ± SE. *t*-test comparison between locations is also reported ($P \leq 0.05$).

PAPER 3

Effect of soil-applied calcium nitrate during periods of active white root growth on the distribution of selected minerals in the tissues of young, potted apple trees after harvest

Abstract

Various internal and external tree factors influence calcium (Ca) uptake and partitioning to the fruit. Synchronizing soil Ca applications with periods of active white root growth is considered to be particularly important. As 'Golden Delicious' apple (*Malus domestica* Borkh.) is known to be susceptible to bitter pit, a commercially important Ca^{2+} deficiency disorder in fruit, a two-year trial was conducted over the 2015/16 and 2016/17 growing seasons to evaluate the effect of calcium nitrate ($Ca(NO_3)_2$) soil applications during periods of active white root growth on the nutrient distribution in the different tissues of young 'Golden Delicious'/M7 apple trees at harvest, focussing on Ca distribution in the fruit. Trees were potted in 25 L plastic containers and fertigated with a low Ca, balanced nutrient solution from planting (September 2015). Additional $Ca(NO_3)_2$ was applied to the soil in summer (November 2015) as a summer-only treatment at 150 g pot^{-1}, in autumn (May 2016) as an autumn-only treatment at 150 g pot^{-1}, and as a split application both in summer and autumn as a summer/autumn treatment at 75 g pot^{-1}. Summer applications were repeated in the 2016/17 season. Additional soil Ca supply was omitted in the control. Fruit Ca, nitrogen (N), phosphorus (P), potassium (K) and magnesium (Ca) concentrations were determined at harvest (120 DAFB) in the second season. At the end of April 2017, trees were destructively harvested for Ca and N analysis. Although fruit Ca concentrations were within the commercial range at harvest, and the N: Ca, K: Ca and (K + Mg): Ca ratios in the fruit fell below the critical levels associated with bitter pit incidence, no significant differences in fruit Ca concentration were found between treatments. A possible explanation is that soil applications in summer of the 2016/17 season were applied too late, possibly after xylem dysfunction in the fruit had occurred, and therefore did not show an effect in the fruit harvested in that season. A resultant increase in Ca concentration was observed in the roots of the summer-only and summer/autumn treatments, and in the stems and new growth (one-year-old shoots and leaves) of the summer-only treatment. Regarding within tree partitioning of Ca content, however, a significantly higher % of total Ca content was found in

the fruit of the autumn-only treatment. These results, thus, suggest that continuous, annual applications of high levels of $Ca(NO_3)_2$ to the soil in both summer and autumn during active root growth may benefit new growth (shoots, leaves and fruit) in spring via early season remobilization of stored Ca from the roots and reserve tissues of the stems.

Keywords: fruit mineral composition; *Malus domestica*; reserves; tree phenology; xylem.

1. Introduction

The seasonal dynamics of calcium (Ca) influx and accumulation in the fruit depends upon a number of internal and external plant factors operating along the soil-to-fruit continuum (Tromp and Van Vuure, 1993; Saure, 2005; De Freitas and Mitcham, 2012; Montanaro et al., 2014a), genotype as one, playing an important role. For 'Cox's Orange Pippin', Wilkinson and Perring (1964) showed that most Ca enters the fruit early in the season, at which stage fruit growth mainly occurs by cell division, i.e. stage I (Lötze and Theron, 2007; Miqueloto et al., 2014). As in kiwifruit (Montanaro et al., 2014a), maximum fruit Ca levels are attained at approximately 2 – 3 weeks after petal fall (Wilkinson and Perring, 1964). A sharp decline during the last few weeks of cell division is followed by a steadier decline from stage II, when fruit growth occurs by both cell division and cell expansion (Lötze and Theron, 2007; Miqueloto et al., 2014), to stage III, when shoot growth ceases and fruit growth continues via cell expansion (Lötze and Theron, 2007; Miqueloto et al., 2014) up to harvest (Wilkinson and Perring, 1964). These findings do not agree with those of Zavalloni et al. (2001) in 'Golden Delicious', 'Fuji' and 'Braeburn' apple. In these cultivars, Ca uptake by the fruit was unanimously shown to be continuous and linear up to harvest. In 'Golden Smoothee' apple, however, Casero et al. (2017) found that while fruit Ca accumulation was continuous up to harvest, it did not follow a linear pattern. A significant increase in Ca was observed from 24 – 66 days after full bloom (DAFB) (stage I) with a prominent peak at around 38 DAFB. During this period, the fruit accumulated approximately 50 % of the total amount of Ca present at harvest. The initial peak was followed by a gradual decline up to the end of shoot growth (approximately 108 DAFB), after which another period of higher intake occurred up to harvest. Similar findings have been reported by others for 'Golden Delicious' (Lötze and Theron, 2006), 'Starkrimson', 'Sansa', 'Pink Lady', 'Senshu', 'Gala', 'Fuji', 'Red General' and 'New Century' apple (Zheng et al., 2006). It has been suggested that the first peak of Ca influx to the fruit is due to the relatively strong sink strength of the developing fruitlets early in the season,

the second peak closer to harvest, due to a decline in shoot competition for nutrients, and the decline in Ca influx to the fruit between stages II and III, due to higher phloem-influx rates of photosynthates and nutrients nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) to the fruit (Lötze and Theron, 2006; Casero et al., 2017). In the last remaining weeks until harvest, Zheng et al. (2006) reported a marked decrease in fruit Ca concentration among cultivars that display relatively high fruit growth rates, e.g. 'Gala', 'Fuji' and 'Red General'. This likely occurs because the fruit continues to expand while Ca import slowly declines (Montanaro et al., 2014a).

In contrast to nutrient elements N, P, K and Mg that may enter the fruit via the xylem and phloem, Ca translocation to the fruit occurs primarily in the xylem (Tagliavini et al., 2000; Dichio et al., 2003; White and Broadley, 2003) and is, therefore, largely dependent on transpirational water flow (Montanaro et al., 2010a; 2014b). As the fruit matures, the surface area to volume ratio declines, the cuticle becomes less water-permeable due to the deposition of cutin and waxes, and the stomata in the outer layer of the fruit either disappear or become physiologically inactive by conversion into lenticels (Tagliavini et al., 2000; Schlegel and Schönherr, 2002; Taylor and Locascio, 2004). The resultant decline in fruit transpiration leads to a progressive decline in xylem influx to the fruit towards the end of the season (Lang, 1990; Bondada et al., 2005; Morandi et al., 2010; Montanaro et al., 2010a, b), whereas phloem influx remains unaffected (Lang, 1990; Lang and Ryan, 1994; Morandi et al., 2012a, b). In developing apricot fruit, Montanaro et al. (2010a) reported that over 50 % of the total Ca content accrued by the fruit early in the season, i.e. < 28 days after fruit set (DAFS), was due to transpiration. Similar observations have been made for apple fruit (Tromp, 1975, 1979; Tromp and Van Vuure, 1993). Because fruit are relatively low-transpiring organs (White and Broadley, 2003; Taylor and Locascio, 2004; Montanaro et al., 2014b), the high transpiration rates of the leaves and shoot tips may redirect Ca influx away from the fruit (Tromp, 1975; Tromp and Van Vuure, 1993; Taylor and Locascio, 2004), especially from stage II of fruit development (Montanaro et al., 2010b). Once Ca is taken up by the leaves, the majority cannot be mobilized and redistributed to the younger tissues or fruits via the phloem (Mengel, 2002; White and Broadley, 2003; Hirschi, 2004; Gilliham et al., 2011). Thus, the weaker reproductive sinks (i.e. the fruit) become dependent on transpiration-driven influx via the xylem for their immediate supply (White and Broadley, 2003; Gilliham et al., 2011; Hocking et al., 2016). This results in typical Ca concentrations of < 0.01 – 0.02 % DM in the fruit and > 0.3 – 0.5 % DM in the new growth (leaves and shoots) at the end of the season (Lüdders, 1980; Vang-Petersen, 1980;

White, 2011). In kiwifruit, Dichio et al. (2003) and Morandi et al. (2009) suggested that the decline in xylem influx to the fruit was rather due to a decrease in xylem hydraulic conductance due to a loss of xylem functionality in the pedicel and/or the fruit during the later stages of fruit development, while Mazzeo et al. (2013) suggested that both a decline in transpiration and a loss in xylem functionality play a role.

The origin of the decline in xylem influx to the fruit may differ in mode and location among fruit types (Knipfer et al., 2015). For instance, in grape, the decline in xylem influx to the fruit is due to a decline in xylem hydraulic conductivity in the pedicel and not the berry, as the vessels in the berry remain functional throughout the growing season (Rogiers et al., 2001; Bondada et al., 2005; Chatelet et al., 2008). In apple, however, the decline in xylem influx to the fruit is not associated with changes in the vasculature of the pedicel as xylem vessels remain largely functional up to harvest (Lang and Ryan, 1994; Dražeta et al., 2004a), but with changes in xylem functionality within the fruit itself (Dražeta et al., 2001, 2004b; Amarante et al., 2013; Miqueloto et al., 2014). Dražeta et al. (2004b), Amarante et al. (2013) and Miqueloto et al. (2014) found near complete disintegration of the xylem vessels in the fruit occurred around 40 – 45 DAFB in ‘Braeburn’ and ‘Catarina’, whereas in ‘Granny Smith’ and ‘Fuji’, the xylem remained intact for much longer; up to 67 DAFB for ‘Granny Smith’ and 80 – 100 DAFB for ‘Fuji’. Le Roux (2018) also confirmed differences in xylem functionality between ‘Golden Delicious’, ‘Granny Smith’, ‘Braeburn’, ‘Fuji’, ‘Cripps Red’ and ‘Cripps Pink’. In ‘Golden Delicious’, substantial xylem disintegration occurred around 56 DAFB. Like apple, the loss of xylem conductance in kiwifruit occurs within the fruit, however, in kiwifruit, xylem disintegration occurs intermittently with periods of functional recovery because of early-season differentiation of new vessels (Dichio et al., 2003). The first observations of possible functional recovery in apple fruit was reported by Le Roux (2018).

The seasonal decline in xylem functionality disrupts Ca import to the fruit, while phloem-influx of nutrient elements N, P, K and Mg continue throughout the growing season (Wilkinson and Perring, 1964; Zavalloni et al., 2001; Zanotelli et al., 2014). In apple orchards in the Vyeboom region of the Western Cape, Wilsdorf (2011) reported a lack of effect on fruit Ca concentration in ‘Braeburn’ at 80 DAFB from early-season soil Ca applications (14 DAFB in the first season and 20 DAFB in the second season). A possible explanation is that young white roots were absent at the time of soil Ca applications in the first two seasons of the study. It is generally accepted that unsuberized, white roots or root tips are primarily responsible for Ca uptake from

the soil (Clarkson, 1984, 1993; Bouma et al., 2001; Volder et al., 2005; De Freitas and Mitcham, 2012; Gu et al., 2015). In contrast to the northern hemisphere where white root growth in apple trees peak in spring (around full-bloom) and trees normally fail to produce new roots during autumn and into winter (Head, 1966, 1967; Psarras et al., 2000; Eissenstat et al., 2006; Yao et al., 2006; Ma et al., 2013), in mature, bearing apple trees in the Grabouw region of the Western Cape, Van Zyl (2016) reported two prominent peaks in white root production. The first peak occurred early in summer (November to December) coincident with a period of rapid shoot growth, and the second, in autumn/winter (March to August) post-harvest. In the same study, Van Zyl (2016) reported active uptake of Ca from soil-applied calcium nitrate ($Ca(NO_3)_2$) in potted, non-bearing 'Golden Delicious'/M7 apple trees. Calcium was mainly concentrated in the new growth (shoots and leaves) following soil applications at the time of the summer peak, and in the roots and stems of the trees following soil applications at the time of the autumn/winter peak. Because the trees did not bear fruit, the influence of different timings of soil Ca applications on fruit Ca levels could not be established. In the third season of the study conducted on 'Braeburn' apple by Wilsdorf (2011), a lack of response to early-season soil Ca applications (30 DAFB) in fruit harvested at 80 DAFB was once again observed, even though soil applications now coincided with a period of active white root growth. The lack of response was ascribed to the early-season decline in xylem functionality in the fruit, as per the findings of Dražeta et al. (2004b). Despite a lack in immediate response, after three consecutive growing seasons, a combination of soil Ca applications at fruit set (late-October/early-November) and after harvest (mid- to late-May) caused a steady increase in fruit Ca concentration, presumably via remobilization of seasonally stored Ca accumulated in the roots and reserve tissues of the trees.

The present study builds on work by Van Zyl (2016) with the addition, where the young, potted 'Golden Delicious'/M7 apple trees were allowed to crop, thus, enabling evaluation of fruit in addition to other plant tissues. The main objective was to determine if Ca uptake and partitioning to the fruit can be increased substantially by applying additional $Ca(NO_3)_2$ to the soil in summer, autumn, or both in summer and autumn during periods of active white root growth.

2. Methods and Materials

2.1. Plant material and experimental design

A potting trail with two-year-old 'Golden Delicious'/M7 apple trees (Stargrow Nursery, Stellenbosch) was conducted on the Welgevallen Experimental Farm (33°56'33"S, 18°51'56"E) of Stellenbosch University in the Western Cape, South Africa, over two consecutive seasons (2015/16 and 2016/17). Trees were planted in a 4:1 (*v/v*) mixture of coarse sand and compost (Builders Express, Stellenbosch) in 25 L (35 cm in diameter) brown plastic pots and fertigated daily with a low Ca, balanced nutrient solution for the duration of the trial, as per the method described in full in Paper 1.

Under conditions of inadequate winter chilling in areas with warm climates, such as Stellenbosch, poor and/or less synchronized bud break in fruit trees can lead to protracted bloom and lower yields (Jacobs et al., 2002). Therefore, each individual tree was treated with a solution of 0.75 % hydrogen cyanamide plus 4 % mineral oil (Sagredo et al., 2005), that was premixed in a tank with water. When most of the buds were either swollen or showed the first signs of budburst at the end of August 2016 (Fig. 1a), the rest breaking solution was sprayed onto each bud using a handgun to the point of run-off.

At full-bloom (first week of October 2016; Fig. 1b), trees were cross-pollinated (by bees) with bouquets of 'Royal Gala' flowers from an orchard at the Welgevallen Experimental Farm to ensure proper fruit set. Five flowering shoots were placed in an open container filled with water. Three to five containers were placed in each row and evenly spaced between the trees (Fig. 1c). After fruit set, all trees were hand thinned to one fruitlet per cluster at the end of October 2016 (28 DAFB; Fig. 1d).

The experimental layout was a randomized complete block design with 12 blocks and four treatments, where individual trees represented experimental units. Treatments included soil applications of $Ca(NO_3)_2$ (YaraLiva Nitrabor®, Yara Africa Fertilizer (Pty) Ltd.) that were split applied by hand in equal quantities. Applications were performed on a weekly basis, over a three-week period, to the surface of each pot in either summer, autumn or as a split application, both in summer and autumn. The control received no additional $Ca(NO_3)_2$. Summer applications commenced on 23 November 2015, and autumn applications on 6 May 2016.

Summer applications were repeated in the second season on 25 November 2016. The summer-only and autumn-only treatments consisted of 150 g $Ca(NO_3)_2$ granules pot^{-1}, respectively, and the summer/autumn treatment of 75 g $Ca(NO_3)_2$ granules pot^{-1} per application. The pots were watered with 2 L of tap water after each application to ensure proper dissolution of the granules. The timing of applications was selected to coincide with periods of active white root growth as quantified by Van Zyl (2016) for mature, bearing 'Golden Delicious'/M793 apple trees in the Grabouw region of the Western Cape.

2.2. Measurements

2.2.1. Soil temperature

To establish whether soil temperatures were conducive to root growth, hourly soil temperatures (°C) were recorded with Tiny-tag data logger soil probes (TPG-4505 Gemini Data Loggers Ltd., Chichester, West Sussex, UK) at a depth of 15 cm below the surface of the growing medium, in two randomly selected pots for the duration of the trial.

2.2.2. White root growth

To confirm whether $Ca(NO_3)_2$ applications to the soil were synchronized with periods of active white root growth, the latter was quantified in relation to above-ground tree phenology using minirhizotrons according to standard procedure (Paper 1). A single tube was installed per tree, totaling eight tubes at two replications per treatment. Observations started three months after installation to allow the growing medium to settle and the roots to grow naturally around the tubes. Root scans were performed on a bi-weekly basis (weather permitting) from January 2016 to April 2017.

2.2.3. Tree height and stem diameter

Tree height (m) and stem diameter (mm) measurements were performed at the start and end of the trial, as described in Paper 1.

2.2.4. Calcium reserve status at planting

Twelve additional trees were chosen at random at the start of the trial and individually separated into roots and stems. All samples were oven-dried at 70 °C until a constant mass was achieved.

Thereafter, dried tissue samples of eight single tree replicates were randomly selected and sent for macro mineral analyses at a commercial laboratory (Bemlab (Pty) Ltd., 16 Van der Berg Crescent, Strand, South Africa, 7140).

2.2.5. Nutrient distribution

Fruit was harvested at pre-optimum maturity (120 DAFB) to prevent theft (Fig. 2). Fruit diameter at harvest was approximately 53 – 59 mm (data not shown). After harvest, the FM of the whole fruit (stalks, core and pips removed) was recorded, then oven-dried at 70 °C until a constant mass was achieved for DM measurements. Thereafter, a subsample of six fruit of comparable size (average DM of 20 g fruit^{-1}, data not shown) of eight single tree replicates per treatment was randomly selected for macro mineral analysis at Bemlab (Pty) Ltd. (16 Van der Berg Crescent, Strand, South Africa, 7140). Given the relationship between bitter pit development in apple and nutrient imbalances in the fruit flesh at harvest (Raese and Staiff, 1990; Amarante et al., 2006a, b, 2013; Casero et al., 2010; De Freitas et al., 2010, 2015; Miqueloto et al., 2014), ratios of N: Ca, K: Ca, (K + Mg): Ca and (K + Mg + N): Ca were also calculated.

All trees were destructively harvested at the end of April 2017 and separated into roots, stems (two- and three-year-old wood) and new growth (one-year-old shoots including spurs and leaves). Roots were washed with water to remove any adhering growing medium. The FM and DM of all the samples were recorded. To quantify nutrient distribution in the different plant parts of the trees after harvest, dried tissue samples of eight single tree replicates per treatment were randomly selected and sent for macro mineral analysis at Bemlab (Pty) Ltd. (16 Van der Berg Crescent, Strand, South Africa, 7140). Concentrations of selected minerals in the fruit are expressed as mg 100 g^{-1} DM. Concentrations of Ca and N in the roots, stems and new growth are expressed as % DM. After converting mg 100 g^{-1} DM to % DM, the Ca content (g DM) in each respective plant part was determined by multiplying the Ca concentration (% DM) with the corresponding DM (g) of each plant part (Annexure 3, Table 1). To determine the percentage distribution of Ca in each plant part (% of total), the Ca content (g DM) of each plant part was divided by the total Ca content (g DM) per tree (Annexure 3, Table 1) and expressed as a percentage.

2.2.6. Calcium concentration of the growing medium

A single composite sample of the growing medium was collected at the start of the trial prior to planting (September 2015) from four randomly selected pots to serve as a reference at planting. At the end of the trial, a single composite sample from four randomly selected pots per treatment were collected for Ca analysis at Bemlab (Pty) Ltd. (16 Van der Berg Crescent, Strand, South Africa, 7140).

2.3. Statiscal analysis

In all analyses, the dependent variables were $\log_{10}$-transformed to achieve homoscedasticity of variances among treatments if they were not. Tree height (m) and stem diameter (mm) among treatments were analysed with analysis of covariance (ANCOVA) using the general linear model (GLM) procedure. Initial measurements at planting were used as covariates. Means were separated using the Least Square Means (LSM) test. Treatment differences were evaluated with a two-way analyses of variance (ANOVA) using the GLM procedure. Means were separated using Fisher's LSD (ordinary pairwise *t*-tests). All data effects were considered significant if $P \leq 0.05$. All statistical analyses were performed with Statistical Analysis System (SAS) version 9.4 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Soil temperature

The average soil temperatures (°C) of two randomly selected pots were plotted against time (months) from January 2016 to January 2017 (Annexure 3, Fig. 1). Soil temperatures were high in summer (January to February 2016, 2017), decreased steadily in autumn (March to April 2016) and reached a minimum in winter (July 2016). The maximum temperature recorded at 15 cm below the surface was 41 °C in January 2016 (38 °C in January 2017), and the minimum temperature, 3 °C in July 2016.

3.2. White root growth

The average white root counts per date (months) in relation to above-ground tree phenology are depicted in Fig. 3. True comparisons of white root growth between treatments were

difficult, as root scans revealed large variations in white root numbers between replications, especially for the autumn treatment, throughout the trial period.

A considerable number of white root tips were present when the trees of the autumn-only and summer/autumn treatments received additional $Ca(NO_3)_2$ soil applications (May 2016; Fig. 3A). White root numbers remained relatively constant from the onset of leaf drop in mid-May 2016 (Fig. 3i) until the end of winter (August 2016). Likewise, from bud break (early-September 2016; Fig. 3iv) until full-bloom (early-October 2016; Fig. 3v), no significant changes in white root growth were observed. Following this period until 50 % shoot extension growth (December 2016; Fig. 3vi), a substantial rise in white root numbers were observed, particularly in the autumn-only treatment. During that period (late-November 2016, ± 56 DAFB), the trees of the summer-only and summer/autumn treatments received additional soil $Ca(NO_3)_2$ applications (Fig. 3B). Thereafter, a slow but steady decline in white numbers were observed in the control, autumn-only and summer/autumn treatments, while white root numbers in the summer-only treatment remained relatively constant until the end of the trial (April 2017).

3.3. Tree height and stem diameter

Treatments that included summer $Ca(NO_3)_2$ applications had significantly larger stem diameters, whereas no significant differences were found for tree height between treatments at tree harvest in April 2017 (Table 1).

3.4. Tree growth and dry mass allocation

No significant differences between treatments were found for the $FM_{(roots)}$ and $DM_{(roots)}$, or for the $FM_{(fruit)}$ and $DM_{(fruit)}$, but significant differences in FM and DM of the stems and new growth were found between treatments (Table 2). Compared to the $FM_{(stems)}$ of the control and autumn-only treatment, which did not differ significantly, a significant increase in $FM_{(stems)}$ was found in the summer-only and summer/autumn treatments, which did not differ significantly. The summer-only treatment had the highest $DM_{(stems)}$, although it was not significantly different from the summer/autumn treatment. The autumn-only treatment had the lowest $DM_{(stems)}$, but this was not significantly lower than the control. The $DM_{(stems)}$ of the summer/autumn treatment did not differ significantly from the control. The summer-only

treatment had the highest $FM_{(new\ growth)}$, although it was not significantly different from the summer/autumn treatment. The $FM_{(new\ growth)}$ of the summer-only and summer/autumn treatments was significantly higher compared to the control, which did not differ significantly from the autumn-only treatment. Between the autumn-only, summer-only and summer/autumn treatments, no significant differences in $DM_{(new\ growth)}$ were found, but the $DM_{(new\ growth)}$ of the treatments that included summer applications were significantly higher compared to the control.

3.5. Nutrient distribution in the roots, stems and new growth

3.5.1. Calcium

Significant differences in Ca concentration were found in the roots, stems and new growth between treatments (Table 3). At the end of April 2017, the $Ca_{[roots]}$ of the control was significantly higher compared to the control at planting. No significant difference was found in the $Ca_{[stems]}$ of the control at planting and after harvest. Although the $Ca_{[roots]}$ of the summer-only and summer/autumn treatments did not differ significantly, both resulted in a significantly higher $Ca_{[roots]}$ compared to the control, while that of the autumn-only treatment did not differ significantly from any of the other treatments or the control. The $Ca_{[stems]}$ of the summer-only treatment was significantly higher compared to the control and autumn-only treatment, which did not differ significantly, while the $Ca_{[stems]}$ of the summer/autumn treatment did not differ significantly from any of the other treatments or the control. Although the $Ca_{[new\ growth]}$ of the summer-only and autumn-only treatments did not differ significantly, both resulted in a significantly higher $Ca_{[new\ growth]}$ compared to the control, while that of the summer/autumn treatment did not differ significantly from any of the other treatments or the control. Within tree partitioning of Ca content between treatments are shown in Fig. 4. At the end of April 2017, no significant differences in % of total Ca content in the roots, stems or new growth were found between the treatments or the control.

3.5.2. Nitrogen

Significant differences in N concentration were found in the roots, stems and new growth between treatments (Table 3). The $N_{[roots]}$ and $N_{[new\ growth]}$ was significantly higher in the treatments that included summer $Ca(NO_3)_2$ applications. Although the $N_{[roots]}$ and $N_{[new\ growth]}$ of the autumn-only treatment was significantly higher compared to the control, it was

significantly lower compared to the treatments that included summer $Ca(NO_3)_2$ applications. The $N_{[stems]}$ were significantly higher in the treatments that included summer $Ca(NO_3)_2$ applications, while that of the autumn-only treatment did not differ significantly from the control.

3.6. Fruit mineral analysis

The Ca concentration as well as the N, P and Mg concentrations of the fruit of the summer-only, autumn-only and summer/autumn treatments did not significantly from each other or the control at 120 DAFB (Table 4). The $K_{[fruit]}$ of the control was significantly higher compared to the summer-only, autumn-only and summer/autumn treatments, which did not differ significantly. Regarding within tree partitioning of Ca content between treatments at the end of the trial (Fig. 4), a significantly higher % of total Ca content was found in the fruit of the autumn-only treatment, while the % of total Ca content allocated to the fruit of the other treatments did not differ significantly from each other or the control. There were no significant differences in the N: Ca and (K + Mg + N): Ca ratios in the fruit between treatments at 120 DAFB (Table 5). The K: Ca and (K + Mg): Ca ratios in the fruit of the control were significantly higher compared to the autumn-only and summer/autumn treatments, which did not differ significantly. The K: Ca and (K + Mg): Ca ratios in the fruit of the summer-only treatment did not differ significantly from the other treatments or the control.

3.7. Calcium concentration of the growing medium

The Ca concentration (cmol(+) kg^{-1}) of the growing medium at planting (September 2015), and of the control, summer-only, autumn-only and summer/autumn treatments at the end of the trial (April 2017), are depicted in Table 6. Due to the sampling method employed, significant differences between treatments could not be established.

4. Discussion

In the 2016/17 season, white root numbers peaked in summer (November/December), despite soil temperatures having risen above the maximum temperature of 35 °C for optimum root growth (Nightingale, 1935). For most of autumn/winter in the first season (2015/2016), soil temperatures remained above the minimum temperature of 7 °C for optimum root growth

(Nightingale, 1935), thus, allowing root growth. Soil $Ca(NO_3)_2$ applications were synchronized with periods of active white root growth in late-autumn/early-winter (May 2016) and summer (November 2016). Unfortunately, it is not known whether active root growth was taking place at the time of the first summer applications (November 2015), as white root growth was not quantified before January 2016. However, soil temperatures (around 38 °C) resembled those recorded in summer of the 2016/17 season.

Treatments that included summer $Ca(NO_3)_2$ soil applications increased tree growth. Compared to the control and autumn-only treatment, a significant increase in stem diameter and new growth (FM) was found. The DM accumulation in the stems and new growth of the summer-only and summer/autumn treatments corresponded well with an increase in N concentration until the end of April 2017. The available N supply may have originated from remobilization of the N reserves in the roots and permanent structural components of the trees (Cheng et al., 2001; Neilsen et al., 2001; Cheng and Fuchigami, 2002; Quartieri et al., 2002; Cheng and Raba, 2009; Millard and Grelet, 2010), where N was stored following N withdrawal from the leaves after shoot growth cessation in both seasons (Titus and Kang, 1982). Additionally, nitrogen may have been remobilized from the leaves during leaf senescence in autumn/winter in the previous season (Titus and Kang, 1982) following summer (November 2015) (Millard and Thompson, 1989; Toselli et al., 2000) as well as autumn $Ca(NO_3)_2$ soil applications (May 2016) in the previous season (Titus and Kang, 1982). Alternatively, N may have been obtained directly from the roots in the current growing season following summer $Ca(NO_3)_2$ soil applications (November 2016) during active shoot growth (Quartieri et al., 2002; Cheng and Raba, 2009; Millard and Grelet, 2010).

Root N uptake of the current season soil supply contributed significantly to the N concentration of the vegetative plant parts of the trees, indicated by the significantly higher N concentration of the roots, stems and new growth of treatments that included summer $Ca(NO_3)_2$ applications. The N concentration of the fruit of the summer-only and summer/autumn treatments, however, did not differ significantly from the autumn-only treatment or the control. Previous studies on pear (Tagliavini et al., 2000), showed that fruit is more dependent on the remobilization of tree reserves for their N supply than the vegetative plant parts of the tree, especially during the early stage of fruit development. Likewise in apple (Cheng et al., 2001; Cheng and Fuchigami, 2002), the utilization of N reserves early in the season was proportional to the total amount of N that accumulated in the roots and reserve tissues of the trees during the previous growing season,

and this occurred independently of the current season soil N supply. In addition, Millard and Grelet (2010) reported that root N uptake for above-ground growth only occurs near or after the cessation of N remobilization from the reserves in the tree. Therefore, it is possible that the contribution of N from the reserves to the fruit was substantial compared to the contribution of root-supplied N from the current season soil supply, in agreement with Quartieri et al. (2002). When $Ca(NO_3)_2$ was applied to the soil in summer, the fruit were possibly relatively weak sinks with respect to N (Tagliavini et al., 2000), as the timing of application concurred with rapid shoot growth. It is, thus, possible that a higher percentage of soil-supplied N was allocated to the shoots and leaves following uptake, while a lower percentage N was allocated to the fruit. This is also evident in the N: Ca and (K + Mg + N): Ca ratios in the fruit at harvest, which did not differ significantly between treatments. It is important to note that the fruit was harvested after shoot growth cessation at the end of January 2017, while the trees were only destructively harvested at the end of April 2017. This allowed more time for N allocation to the roots, stems and new growth. The significantly higher N concentration of the roots and stems of the summer-only and summer/autumn treatments at the end of April 2017, possibly indicates that the current season N supply contributed positively towards N storage, confirming previous findings (Millard and Thompson, 1989; Toselli et al., 2000).

Remobilization of previously absorbed nutrients early in the next season is not limited to N (Terblanche, 1972; Terblanche et al., 1979; Tromp, 1983; Kangueehi, 2008). Up to 9 % of Ca taken up by the roots may become fixed, either as oxalate crystals, or by structural incorporation in the bark and woody tissues of trees (Ferguson and Bollard, 1976; Terblanche et al., 1979; Ferguson, 1980), but a greater portion of root-supplied Ca may be available for redistribution, the latter said reserves, reversibly retained by adsorption on exchange sites in the free space of the roots and woody tissues of trees (Ferguson and Clarkson, 1976; Ferguson and Bollard, 1976; Ferguson, 1980). In apple, it is this water-soluble or easily extractable Ca fraction that can contribute up to 25 % of the total Ca content in the new growth (leaves, shoots and fruit) via remobilization the following spring (Terblanche et al., 1979).

The average Ca concentration in the flesh of apple fruit at harvest normally ranges from 3 – 6 mg 100 g^{-1} FM or 25 – 35 mg 100 g^{-1} DM (Zavalloni et al., 2001; Saure, 2005). Despite the trees in the present study having received additional soil Ca applications in summer, autumn or both in summer and autumn in the previous growing season (2015/16), plus additional summer applications in the current season (2016/17), fruit Ca concentrations at harvest

remained within the normal range, but the N: Ca, K: Ca and (K + Mg): Ca ratios in the fruit fell below the critical levels associated with bitter pit incidence (Sharples, 1980; Raese and Staiff, 1990; Amarante et al., 2012; De Freitas et al., 2015). Although no significant differences in fruit Ca concentration were found between treatments, a significantly higher % of total Ca content was found in the fruit of the autumn-only treatment (2 % vs 1 % in the control, summer-only and summer/autumn treatments).

As soil Ca applications coincided with periods of active white root growth in both seasons, the overall lack of response to soil Ca supply in the fruit of the summer-only and summer/autumn treatments cannot be attributed to an incorrect timing of soil Ca supply, as suggested previously by Wilsdorf (2011). Factors other than timing of soil Ca supply should therefore be considered for the lack of response at harvest. According to Saure (2005), fruit thinning reduces fruit Ca concentration by dilution through an increase in fruit size. Although fruit growth dynamics and the total number of fruit at harvest between treatments were not quantified in the current experiment, we assume fruit growth rates between treatments were similar, as fruit size and fruit Ca concentrations between treatments at harvest were similar as well. Thus, another possible explanation for the lack of response in fruit Ca levels to additional soil applications in summer, may be a loss in xylem functionality in the fruit and a resultant decline in Ca import to the fruit via root uptake at that time (Dražeta et al., 2004b, Amarante et al., 2013; Miqueloto et al., 2014; Le Roux, 2018). Xylem conductance was, however, not quantified in this study. Lastly, whilst the Ca concentration of the new growth (one-year-old shoots and leaves) was significantly higher in the summer-only treatment compared to the control and that of the summer/autumn treatment did not differ significantly from the summer-only treatment or the control at the end of April 2017, the lack of response in fruit Ca levels to additional soil applications in summer in the current season indicates the predominant role of Ca reserve accumulation in the trees in supporting new growth (particularly the fruit) early in the next season, as reported by Wilsdorf (2011). The significantly higher Ca concentration of the roots of the summer-only and summer/autumn treatments, and the significantly higher Ca concentration of the stems of the summer-only treatment at the end of April 2017, also endorse this hypothesis.

In contrast to previous findings (Van Zyl, 2016), results from Paper 1 in this study conducted on similar trees under the same conditions, showed that a relatively high concentration of $Ca(NO_3)_2$ (150 g pot^{-1}) applied to the soil in autumn (May 2016) was primarily allocated to the

leaves towards the end of winter following an extended leaf drop period. Normal leaf abscission, likewise, occurred late during winter in the current experiment. As the total Ca content per tree (Annexure 3, Table 1) in the autumn-only treatment (5.38 g DM) was significantly lower than the summer-only (7.95 g DM) and summer/autumn treatments (7.43 g DM), a sizable fraction of total Ca uptake in trees of the autumn-only treatment was probably first allocated to the still metabolic active leaves (Van Zyl, 2016), and then lost via leaf abscission during winter. However, since a significantly higher % of total Ca content was found in the fruit of the autumn-only treatment, the fraction of available Ca that was taken up by the roots and stored as reserves towards the end of the previous growing season (2015/16) may have been sufficient to support fruit growth and development early in the following season (2016/17). In support, the Ca concentration of the roots and stems of the autumn-only treatment did not differ significantly from the control at the end of April 2017. Since the % of total Ca content in the fruit of the summer/autumn treatment did not differ significantly from the control and was significantly lower than that of the autumn-only treatment, under local conditions of insufficient winter chilling, it seems a positive response in fruit Ca levels to additional $Ca(NO_3)_2$ soil applications in autumn during active root growth depends on the rate of application. Moreover, since the Ca concentration of the new growth of the summer-only and autumn-only treatments was significantly higher compared to the control, while that of summer/autumn treatment did not differ significantly from the control, the current strategy of synchronizing high rates of additional soil Ca supply with periods of active white root growth in ‘Golden Delicious’ apple, both in summer and autumn, may benefit new growth (shoots, leaves and fruit) early in the next season via remobilization of stored Ca from the roots and reserve tissues of the stems.

5. Conclusion

In contrast to previous findings in potted, non-bearing ‘Golden Delicious’/M7 apple trees in the Western Cape, the addition of soil Ca supply in summer, autumn or both in summer and autumn had little influence on the partitioning of Ca content in the roots, stems and new growth between treatments after two growing seasons. A significantly higher % of total Ca content was, however, allocated to the fruit of the autumn-only treatment. Summer $Ca(NO_3)_2$ applications did not substantially increase fruit Ca levels at harvest, but a significant increase in Ca concentration in the new growth confirms primary xylem transport to the more dominant leaf and shoot sinks in the second season (2016/17). The possible early disintegration of xylem

vessels in the fruit at this time, could have furthermore restricted Ca influx to the fruit. At the end of April 2017, treatments that included summer applications significantly increased the Ca concentration of the roots and stems. The effect of the summer/autumn treatment was like that of the autumn-only treatment, while the summer-only treatment had the greatest effect.

Under sub-optimal winter chilling conditions, an extended leaf drop period following soil $Ca(NO_3)_2$ applications in autumn did not have a negative impact on Ca allocation to the new growth (including the fruit) in the following season. Although a substantial fraction of total Ca uptake may have been lost through transport to the leaves during autumn and into winter, Ca reserves, as accumulated in the roots and stems following high $Ca(NO_3)_2$ soil applications in autumn, was sufficient to support new growth (including the fruit) early in the following season. However, since the % of total Ca content in the fruit of the summer/autumn treatment did not differ significantly from the control and was significantly lower than that of the autumn-only treatment, the advantage of soil-applied Ca in autumn/early-winter during active root growth seems to depend on the rate of application.

In locally established 'Golden Delicious' apple trees, the results of this experiment suggest that relatively high rates of soil-applied $Ca(NO_3)_2$, both in summer and autumn during active root growth, may benefit the following season's crop through remobilization of stored Ca in the roots and reserve tissues of the stems. However, as fruit Ca concentrations at harvest did not differ significantly between treatments after two seasons, the long-term benefits of employing such a strategy under local conditions needs to be confirmed. A longer trial period is therefore advised.

Acknowledgements

Yara Africa Fertilizer (Pty). Ltd. for their support and funding of this trial.

References

Amarante, C.V.T., Chaves, D.V., Ernani, P.R., 2006a. Mineral composition and bitter pit severity in 'Catarina' apples. Rev. Bras. Frutic. 28, 51–54.

Amarante, C.V.T., Chaves, D.V., Ernani, P.R., 2006b. Multivariate analysis of nutritional attributes associated with bitter pit in 'Gala' apples. Pesq. Agropec. Bras. 41, 841–846.

Amarante, C.V.T., Miqueloto, A., Steffens, C.A., dos Santos, A., Argenta, L.C., 2013. Changes in xylem functionality during apple fruit development: Implications on calcium concentration and incidence of bitter pit. Acta Hort. 1012, 135–140.

Amarante, C.V.T., Steffens, C.A., Ernani, P.R., Argenta, L.C., 2012. Assessment of bitter pit risk in 'Gala' apples by fruit infiltration with magnesium. Acta Hort. 934, 855–860.

Bondada, B.R., Matthews, M.A., Shackel, K.A., 2005. Functional xylem in the post-veraison grape berry. J. Exp. Bot. 56, 2949–2957.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Casero, T., Benavides, A.L., Recasens, I., 2010. Interrelation between fruit mineral content and pre-harvest calcium treatments on 'Golden Smoothee' apple quality. J. Plant Nutr. 33, 27–37.

Casero, T., Torres, E., Alegre, S., Recasens, I., 2017. Macronutrient accumulation dynamics in apple fruits. J. Plant Nutr. 40, 2468–2476.

Chatelet, D.S., Rost, T.L., Shackel, K.A., Matthews, M.A., 2008. The peripheral xylem of grapevine (*Vitis vinifera*). 1. Structural integrity in post-veraison berries. J. Exp. Bot. 59, 1987–1996.

Cheng, L., Dong, S., Guak, S., Fuchigami, L.H., 2001. Effects of nitrogen fertigation on reserve nitrogen and carbohydrate status and regrowth performance of pear nursery plants. Acta Hort. 564, 51–62.

Cheng, L., Fuchigami, L.H., 2002. Growth of young apple trees in relation to reserve nitrogen and carbohydrates. Tree Physiol. 22, 1297–1303.

Cheng, L., Raba, R., 2009. Accumulation of macro- and micronutrients and nitrogen demand-supply relationship of 'Gala'/'Malling 26' apple trees grown in sand culture. J. Am. Soc.

Hort. Sci. 134, 3–13.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

De Freitas, S.T., Amarante, C.V.T., Labavitch, J.M., Mitcham, E.J., 2010. Cellular approach to understand bitter pit development in apple fruit. Postharvest Biol. Technol. 57, 6–13.

De Freitas, S.T., Amarante, C.V.T., Mitcham, E.J., 2015. Mechanisms regulating apple cultivar susceptibility to bitter pit. Sci. Hort. 186, 54–60.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hort. Rev. 40, 107–146.

Dichio, B., Remorini, D., Lang, S., 2003. Developmental changes in xylem functionality in kiwifruit fruit: Implications for fruit calcium accumulation. Acta Hort. 610, 191–195.

Dražeta, L., Lang, A., Cappellini, C., Hall, A.J., Volz, R.K., Jameson, P.E., 2004a. Vessel differentiation in the pedicel of apple and the effects of auxin transport inhibition. Physiol. Plant. 120, 162–170.

Dražeta, L., Lang, A., Hall, A.J., Volz, R.K., Jameson, P.E., 2004b. Causes and effects of changes in xylem functionality in apple fruit. Ann. Bot. 93, 375–382.

Dražeta, L., Lang, A., Morgan, L., Volz, R., Jameson, P.E., 2001. Bitter pit and vascular function in apples. Acta Hort. 564, 387–392.

Eissenstat, D.M., Bauerle, T.L., Comas, L.H., Neilsen, D., Neilsen, G.H., Lakso, A.N., Smart, D.R., 2006. Seasonal patterns of root growth in relation to shoot phenology in grape and apple. Acta Hort. 721, 21–26.

Ferguson, I.B., 1980. The uptake and transport of calcium in the fruit tree, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 183–192.

Ferguson, I.B., Bollard, E.G., 1976. The movement of calcium in woody stems. Ann. Bot. 40, 1057–1065.

Ferguson, I.B., Clarkson, D.T., 1976. Simultaneous uptake and translocation of magnesium and calcium in barley (*Hordeum vulgare* L.) roots. Planta 128, 267–269.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Gu, J., Wei, X., Wang, J., Dong, X., Wang, Z., 2015. Marked differences in standing biomass, length density, anatomy and physiological activity between white and brown roots in *Fraxinus mandshurica* Rupr. plantation. Plant Soil 392, 267–277.

Head, G.C., 1966. Estimating seasonal changes in the quantity of white suberized root on fruit trees. J. Hort. Sci. 41, 197–206.

Head, G.C., 1967. Effects of seasonal changes in shoot growth on the amount of unsuberized root on apple and plum trees. J. Hort. Sci. 42, 169–180.

Hirschi, K.D., 2004. The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol. 136, 2438–2442.

Hocking, B., Tyerman, S.D., Burton, R.A., Gilliham, M., 2016. Fruit Calcium: Transport and Physiology. Front. Plant Sci. 7, 1–17.

Jacobs, J.N., Jacobs, G., Cook, N.C., 2002. Chilling period influences the progression of bud dormancy more than does chilling temperature in apple and pear shoots. J. Hort. Sci. Biotechnol. 77, 333–339.

Kangueehi, G.N., 2008. Nutrient requirement and distribution of intensively grown 'Brookfield Gala' apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Knipfer, T., Fei, J., Gambetta, G.A., McElrone, A.J., Shackel, K.A., Matthews, M.A., 2015. Water transport properties of the grape pedicel during fruit development: Insights into xylem anatomy and function using microtomography. Plant Physiol. 168, 1590–1602.

Lang, A., 1990. Xylem, phloem and transpiration flows in developing apple fruits. J. Exp. Bot. 41, 645–651.

Lang, A., Ryan, K.G., 1994. Vascular development and sap flow in apple pedicels. Ann. Bot. 74, 381–388.

Le Roux, E., 2018. Ca-metalosate as an alternative Ca formulation for decreasing Ca related disorders in fruit trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Lötze, E., Theron, K.I., 2006. Dynamics of calcium uptake with pre-harvest sprays to reduce bitter pit in 'Golden Delicious'. Acta Hort. 721, 313–319.

Lötze, E., Theron, K.I., 2007. Evaluating the effectiveness of pre-harvest calcium applications for bitter pit control in 'Golden Delicious' apples under South African conditions. J. Plant Nutr. 30, 471–485.

Lüdders, P., 1980. Effects of time and amount of nutrient additives on nutrient status and distribution and on fruit quality, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 165–172.

Ma, L., Hou, C.W., Zhang, X.Z., Li, H.L., Han, D., Wang, Y., Han, Z.H., 2013. Seasonal growth and spatial distribution of apple tree roots on different rootstocks or interstems. J. Amer. Soc. Hort. Sci. 138, 79–87.

Mazzeo, M., Dichio, B., Clearwater, M.J., Montanaro, G., Xiloyannis, C., 2013. Hydraulic resistance of developing *Actinidia fruit*. Ann. Bot. 112, 197–205.

Mengel, K., 2002. Alternative or complementary role of foliar supply in mineral nutrition. Acta Hort. 594, 33–47.

Millard, P., Grelet, G., 2010. Nitrogen storage and remobilization by trees: Ecophysiological relevance in a changing world. Tree Physiol. 30, 1083–1095.

Millard, P., Thomson, C.M., 1989. The effect of the autumn senescence of leaves on the internal cycling of nitrogen for the spring growth of apple trees. J. Exp. Bot. 40, 1285–1289.

Miqueloto, A., Amarante, C.V.T., Steffens, C.A., dos Santos, A., Mitcham, E., 2014. Relationship between xylem functionality, calcium content and the incidence of bitter pit in apple fruit. Sci. Hort. 165, 319–323.

Montanaro, G., Dichio, B., Lang, A., Mininni, A.N., Nuzzo, V., Clearwater, M.J., Xiloyannis, C., 2014a. Internal versus external control of calcium nutrition in kiwifruit. J. Plant Nutr. Soil Sci. 177, 819–830.

Montanaro, G., Dichio, B., Xiloyannis, C., 2010a. Significance of fruit transpiration on calcium nutrition in developing apricot fruit. J. Plant Nutr. Soil Sci. 173, 618–622.

Montanaro, G., Tataranni, G., Mazzeo, M., Dichio, B., 2010b. Transpiration and calcium accumulation in apricot fruit. Acta Hort. 862, 429–432.

Montanaro, G., Xiloyannis, C., Dichio, B., Lang, A., 2014b. Fruit microenvironment and fruit

transpiration: Implications for fruit quality traits. Acta Hort. 1038, 171–176.

Morandi, B., Losciale, P., Manfrini, L., Pierpaoli, E., Zibordi, M., Grappadelli, L.C., 2012a. Short-period changes in weather conditions affect xylem, but not phloem flows to young kiwifruit (*Actinidia deliciosa*) berries. Sci. Hort. 142, 74–83.

Morandi, B., Losciale, P., Manfrini, L., Zibordi, M., Pierpaoli, E., Corelli Grappadelli, L., 2012b. How changes in weather conditions affect growth, vascular and transpiration flows in young apple fruit. Acta Hort. 951, 139–146.

Morandi, B., Manfrini, L., Losciale, P., Zibordi, M., Grappadelli, L.C., 2010. Changes in vascular and transpiration flows affect the seasonal and daily growth of kiwifruit (*Actinidia deliciosa*) berry. Ann. Bot. 105, 913–923.

Morandi, B., Manfrini, L., Losciale, P., Zibordi, M., Grappadelli, L.C., 2009. Is late stage reduction of kiwifruit sap flow due to loss of xylem functionality? Acta Hort. 846, 249–254.

Neilsen, D., Millard, P., Neilsen, G.H., Hogue, E.J., 2001. Nitrogen uptake, efficiency of use, and partitioning for growth in young apple trees. J. Am. Soc. Hort. Sci. 126, 144–150.

Nightingale, G.T., 1935. Effects of temperature on growth, anatomy, and metabolism of apple and peach roots. Bot. Gaz. 96, 581–639.

Psarras, G., Merwin, I.A., Lakso, A.N., Ray, J.A., 2000. Root growth phenology, root longevity, and rhizosphere respiration of field grown 'Mutsu' apple trees on 'Malling 9' rootstock. J. Am. Soc. Hort. Sci. 125, 596–602.

Quartieri, M., Millard, P., Tagliavini, M., 2002. Storage and remobilisation of nitrogen by pear (*Pyrus communis* L.) trees as affected by timing of N supply. Eur. J. Agron. 17, 105–110.

Raese, J.T., Staiff, D.C., 1990. Fruit calcium, quality and disorders of apples (*Malus domestica*) and pears (*Pyrus communis*) influenced by fertilizers, in: Van Beusichem, M.L. (Ed.), Plant Nutrition - Physiology and Applications. Kluwer Academic Publishers, Dordrecht, pp. 619–623.

Rogiers, S.Y., Smith, J.A., White, R., Keller, M., Holzapfel, B.P., Virgona, J.M., 2001. Vascular function in berries of *Vitis vinifera* (L) cv. Shiraz. Aust. J. Grape Wine Res. 7, 47–51.

Sagredo, K.X., Theron, K.I., Cook, N.C., 2005. Effect of mineral oil and hydrogen cyanamide

concentration on dormancy breaking in 'Golden Delicious' apple trees. South African J. Plant Soil 22, 251–256.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: Its mechanism and endogenous control. Sci. Hort. 105, 65–89.

Schlegel, T.K., Schönherr, J., 2002. Stage of development affects penetration of calcium chloride into apple fruits. J. Plant Nutr. Soil Sci. 165, 738–745.

Sharples, R.O., 1980. The influence of orchard nutrition on the storage quality of apples and pears grown in the United Kingdom, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 17–28.

Tagliavini, M., Zavalloni, C., Rombola, A.D., Quartieri, M., Malaguti, D., Mazzanti, F., Millard, P., Marangoni, B., 2000. Mineral nutrient partitioning to fruits of deciduous trees. Acta Hort. 512, 131–140.

Taylor, M.D., Locascio, S.J., 2004. Blossom-end rot: A calcium deficiency. J. Plant Nutr. 27, 123–139.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Titus, J.S., Kang, S., 1982. Nitrogen metabolism, translocation, and recycling in apple trees. Hort. Rev. 4, 204–246.

Toselli, M., Flore, J.A., Zavalloni, C., Marangoni, B., 2000. Nitrogen partitioning in apple trees as affected by application time. Horttechnology 10, 136–141.

Tromp, J., 1975. The effect of temperature on growth and mineral nutrition of fruits of apple, with special reference to calcium. Physiol. Plant 33, 87–93.

Tromp, J., 1979. The intake curve for calcium into apple fruits under various environmental conditions. Commun. Soil Sci. Plant Anal. 10, 325–335.

Tromp, J., 1983. Nutrient reserves in roots of fruit trees, in particular carbohydrates and

nitrogen. Plant Soil 71, 401–413.

Tromp, J., Van Vuure, J., 1993. Accumulation of calcium, potassium and magnesium in apple fruits under various conditions of humidity. Physiol. Plant. 89, 149–156.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Vang-Petersen, O., 1980. Calcium nutrition of apple trees: A review. Sci. Hort. 12, 1–9.

Volder, A., Smart, D.R., Bloom, A.J., Eissenstat, D.M., 2005. Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: Implications for root efficiency and competitive effectiveness. New Phytol. 165, 493–501.

White, P.J., 2011. Long-distance Transport in the Xylem and Phloem, Marschner's Mineral Nutrition of Higher Plants: Third Edition. Elsevier Ltd. doi:10.1016/B978-0-12-384905-2.00003-0.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilkinson, B.G., Perring, M.A., 1964. Changes in the chemical composition of apples during development, and near picking time. J. Sci. Food Agric. 15, 146–152.

Wilsdorf, R., 2011. Evaluating the seasonal changes in calcium concentration and distribution in apple fruit after application of diferent calcium fertilisation strategies. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Yao, S., Merwin, I.A., Brown, M.G., 2006. Root dynamics of apple rootstocks in a replanted orchard. HortScience 41, 1149–1155.

Zanotelli, D., Rechenmacher, M., Guerra, W., Cassar, A., Tagliavini, M., 2014. Seasonal uptake rate dynamics and partitioning of mineral nutrients by bourse shoots of field-grown apple trees. Eur. J. Hort. Sci. 79, 203–211.

Zavalloni, C., Marangoni, B., Tagliavini, M., Scudellari, D., 2001. Dynamics of uptake of calcium, potassium and magnesium into apple fruit in a high-density planting. Acta Hort. 564, 113–121.

Zheng, W., You, C., Du, Z., Zhai, H., 2006. Dynamic changes in the calcium content of several apple cultivars during the growing season. Agric. Sci. China 5, 933–937.

Table 1. Main stem diameter (mm) and tree height (m) of three-year-old, potted, 'Golden Delicious'/M7 apple trees in April 2017 in response to different timings of $Ca(NO_3)_2$ soil applications, after consideration of the covariate (initial measurements at planting in September 2015).

Treatment	Diameter (mm)	Length (m)
Control (None)	21.00 b	1.55 ns
Summer [a]	23.88 a	1.56
Autumn [b]	20.83 b	1.53
Summer/Autumn [c]	24.98 a	1.53
P-value	0.0065	0.9875

Adjusted means with the same letters are not significantly different at $P \leq 0.05$.

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Table 2. Fresh mass (g) and dry mass (g) of the roots, stems (two- and three-year-old wood), new growth (one-year-old shoots including spurs and leaves) and fruit (stalks, core and pips removed) of three-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees in response to different timings of $Ca(NO_3)_2$ soil applications. Fruit was harvested at the end of January 2017 (120 DAFB). Trees were destructively harvested at the end of April 2017.

Treatment	Fresh mass (g)				Dry mass (g)			
	Roots	Stems	New growth	Fruit	Roots	Stems	New growth	Fruit
Control (None)	613.25 ns	522.44 b	184.37 c	1125.30 ns	272.88 ns	302.44 bc	94.16 b	225.44 ns
Summer [a]	703.56	724.88 a	291.47 a	1508.90	301.75	408.56 a	146.35 a	301.25
Autumn [b]	575.81	483.00 b	222.27 bc	1350.90	272.31	278.44 c	115.73 ab	254.81
Summer/Autumn [c]	654.81	695.38 a	265.31 ab	1321.40	278.63	393.44 ab	136.28 a	253.69
P-value	0.6047	0.0094	0.0092	0.7932	0.8994	0.0214	0.0086	0.6703

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Table 3. Calcium concentration (% DM) of the roots and stems of the control trees at planting (September 2015). Calcium and nitrogen concentrations (% DM) of the roots, stems (two- and three-year-old wood) and new growth (one-year-old shoots incl. leaves, spurs and leaves) of three-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees in response to different timings of $Ca(NO_3)_2$ soil applications, after destructive harvesting at the end of April 2017.

Treatment	Calcium (% DM)			Nitrogen (% DM)		
	Roots	Stems	New growth	Roots	Stems	New growth
Control (at planting)	0.35 c	0.75 b				
After harvest						
Control (None)	0.49 b	0.69 b	1.53 b	0.41 c	0.53b	0.75 c
Summer [a]	0.59 a	0.93 a	1.93 a	0.84 a	0.91 a	1.37 a
Autumn [b]	0.52 ab	0.72 b	1.81 a	0.58 b	0.64 b	1.01 b
Summer/Autumn [c]	0.57 a	0.84 ab	1.75 ab	0.88 a	0.89 a	1.36 a
P-value	< 0.0001	0.0355	0.0162	< 0.0001	0.0023	< 0.0001

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Table 4. Calcium (Ca), nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) concentrations (mg 100 g^{-1} DM) of the fruit (stalks, core and pips removed) of three-year-old, potted, 'Golden Delicious'/M7 apple trees in response to different timings of $Ca(NO_3)_2$ soil applications, at harvest at the end of January 2017 (120 DAFB).

Treatment	Ca	N	P	K	Mg
Control (None)	30.5 ns	276.6 ns	71.2 ns	692.0 a	31.6 ns
Summer [a]	31.4	283.5	70.3	608.1 b	33.4
Autumn [b]	32.7	277.4	73.6	576.6 b	32.5
Summer/Autumn [c]	36.1	408.1	70.9	564.6 b	34.0
P-value	0.3619	0.4092	0.7761	0.0020	0.3918

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Table 5. Nutrient ratios in the fruit (stalks, core and pips removed) of three-year-old, potted, 'Golden Delicious'/M7 apple trees in response to different timings of $Ca(NO_3)_2$ soil applications, at harvest at the end of January 2017 (120 DAFB).

Treatment	N: Ca	K: Ca	(K + Mg): Ca	(K + Mg + N): Ca
Control (None)	10.0 ns	23.2 a	24.2 a	34.2 ns
Summer [a]	9.0	19.5 ab	20.6 ab	29.6
Autumn [b]	8.2	18.2 b	19.3 b	27.4
Summer/Autumn [c]	11.4	16.2 b	17.2 b	28.6
P-value	0.6989	0.0066	0.0078	0.2626

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Table 6. Calcium concentration (cmol(+) kg^{-1}) of the growing medium at planting (September 2015) and at the end of the trial (April 2017) per treatment. Data was not analysed statistically.

Treatment	Calcium (cmol(+) kg^{-1})
Growing medium at planting	7.87
Control (None)	8.45
Summer [a]	6.78
Autumn [b]	6.14
Summer/Autumn [c]	4.80

[a] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[c] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

Fig. 1. Potted, ‘Golden Delicious’/M7 apple trees sprayed with 0.75 % hydrogen cyanamide plus 4 % mineral oil at the time of bud swell (late-August 2016) (a), trees in full-bloom in the first week of October 2016 (b), ‘Royal Gala’ flowering shoots placed in containers filled with water between the trees at full-bloom to facilitate cross-pollination and ensure proper fruit set (c), trees hand-thinned to one fruitlet per cluster at the end of October (28 DAFB) (d).

Fig. 2. Fruit of three-year-old, potted, ‘Golden Delicious’/M7 apple trees on the day of harvest at the end of January 2017 (120 DAFB, 3 – 4 weeks prior to optimum harvest).

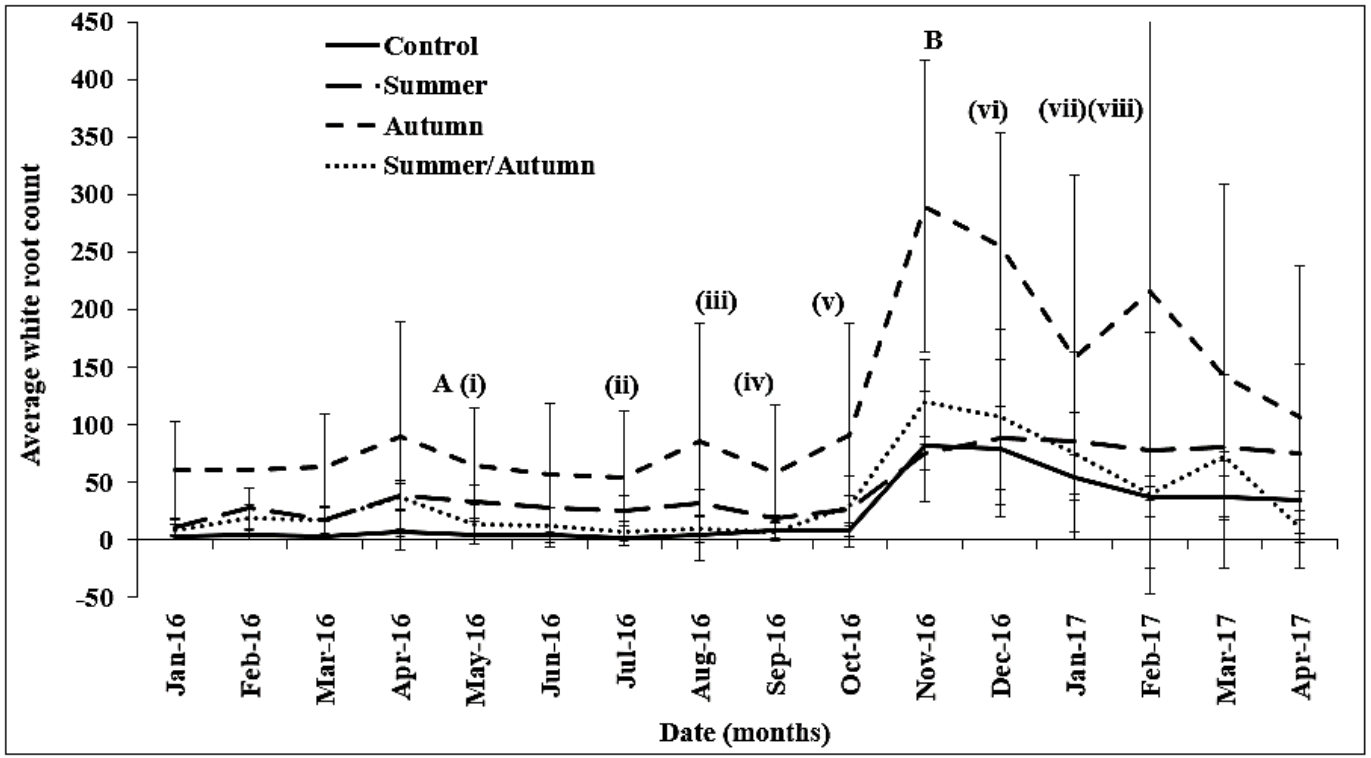
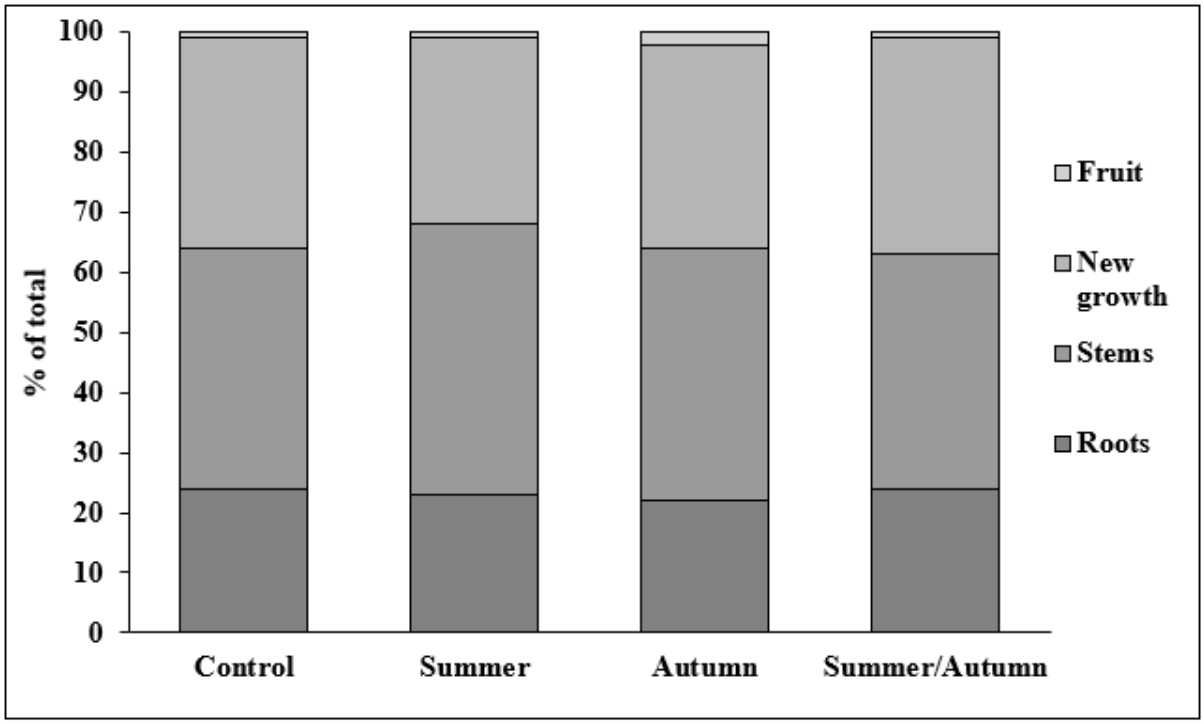


Fig. 3. Average white root counts of potted, 'Golden Delicious'/M7 apple trees recorded from January 2016 to April 2017. Treatments include the control, summer-only, autumn-only, and both summer and autumn $Ca(NO_3)_2$ soil applications, where (A) represents the date of autumn applications (early-May) and (B), the date of the second round of summer applications (late-November 2016). The first round of summer applications in late-November 2015 is not indicated on the graph. The phenological growth stages are indicated as: (i) onset of leaf drop (mid-May 2016), (ii) 50 % leaf drop (mid-July 2016), (iii) end of leaf drop (August 2016), (iv) bud break (late-August/early-September 2016), (v) full-bloom (early-October 2016), (vi) extension growth of 50 % of shoots completed (early-December 2016), (vii) end of shoot extension growth (late-January 2017), and (viii) harvesting of fruit (end of January 2017). Trees were destructively harvested at the end of April 2017. Vertical bars denote standard errors of the mean counts of two replications per treatment.



Treatment	Roots	Stems	New growth	Fruit
Control (None)	25 ns	40 ns	35 ns	1 b
Summer [a]	23	45	31	1 b
Autumn [b]	22	42	34	2 a
Summer/Autumn [c]	24	39	36	1 b
P-value	0.7645	0.2739	0.2333	0.0209

Fig. 4. Partitioning (% of total) of Ca content within two-year old, potted, non-bearing 'Golden Delicious'/M7 apple trees. Fruit was harvested at the end of January 2017. Trees were destructively sampled into roots, stems (two- and three-year-old wood) and new growth (one-year-old shoots incl. spurs and leaves) at the end of April 2017. Treatments include the control (no additional $Ca(NO_3)_2$), summer-only (150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16), autumn-only (150 g $Ca(NO_3)_2$ pot^{-1} in May 2016) and both summer and autumn (75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016) soil applications. Percentage of total Ca content in each plant part (% of total) = (Ca content (g DM) in each respective plant part (Annexure 3, Table 1)/(Total Ca content (g DM) per tree) × 100. Means with the same letters are not significantly different at $P \leq 0.05$.

GENERAL DISCUSSION AND CONCLUSIONS

In apple, fruit physiological disorders related to localized calcium (Ca) deficiency such as bitter pit, are a common phenomenon worldwide and can lead to large losses of marketable yield (Saure, 2005; Hewett, 2006; Jemrić et al., 2016). To reduce the incidence of bitter pit in 'Golden Delicious' apples, studies evaluating the effectiveness of preharvest foliar Ca applications in increasing apple fruit Ca levels to control bitter pit development under South African conditions have achieved various levels of success (Lötze and Theron, 2006, 2007; Lötze et al., 2008). Although it is known that early-season remobilization of available Ca reserves stored in the roots and woody tissues of apple trees following soil Ca uptake can contribute substantially to the total Ca content in the new growth, including the fruit (Terblanche, 1972; Terblanche et al., 1979), little is known about the efficiency of Ca sources applied to the soil during periods of active root growth on Ca reserve replenishment in apple trees established in the warmer growing regions of South Africa. The aim of this study was thus to quantify soil-applied Ca uptake and distribution in relation to periods of active white root growth in young non-bearing 'Golden Delicious'/M7 apple trees in the Western Cape, South Africa.

Trees were planted in sand-compost-filled pots and fertigated with a low Ca, balanced nutrient solution over two consecutive seasons (2015/16 and 2016/17). Three trials were conducted. The main objective of the first trial was to evaluate the impact of increasing levels of soil-applied Ca and autumn defoliation on root growth and Ca partitioning in two-year-old, non-bearing apple trees during winter in the first season. The main objective of the second trial was to quantify Ca uptake and distribution along the length of apple root tips during winter using scanning electron microscopy along with wavelength-dispersive x-ray spectroscopy (SEM-WDS), and of the third trial, to evaluate the impact of soil-applied Ca during periods of active white root growth on Ca uptake and distribution in the different tissues of three-year-old, bearing apple trees, after harvest, in the second season.

At the beginning of the second root flush, in winter (May 2016), calcium nitrate ($Ca(NO_3)_2$) was applied to the soil at three different rates: no additional Ca (control), moderate Ca (1X) and high Ca (2X) to trees that dropped their leaves naturally (NLD) and high Ca (2X) to trees that were manually defoliated (AD) in mid-autumn (April 2016). The relative effect of leaf

transpiration (sap flow) on Ca reserve accumulation in apple trees at the end of winter was monitored with electronic dendrometers on trees from the $2X_{(NLD)}$ and $2X_{(AD)}$ treatments.

An increase in autumn soil Ca supply did not have a significant effect on white root growth during winter, as white root numbers, quantified using minirhizotrons, differed little between the NLD treatments. This contrasts with previous findings (Emanuelsson, 1984; Poovaiah and Reddy, 1991), indicating that a concurrent increase in NO_3^--N supply possibly had a growth suppressing effect on white roots shortly after their emergence, as also demonstrated by Zhang et al. (2007). Despite soil temperature and moisture levels being conducive to root growth during winter (Nightingale, 1935), early leaf loss via defoliation in autumn resulted in a marked decline in white root numbers that remained low for the rest of winter, in agreement with Maggs (1965) and Head (1969) in apple. In the NLD treatments, where most trees reached 50 % leaf drop late in winter (July 2016), a considerable number of young white roots were visible throughout the trial period, i.e. until the end of August 2016. This suggests that new root growth may require some stimulus from the growing shoot. Indeed, Van Zyl (2016) reported a steady supply of current photosynthates to the roots of apple trees at least until 50 % leaf drop.

Despite the drastic decline in white root numbers in the AD trees during winter, root Ca concentration of the $2X_{(AD)}$ treatment was significantly higher compared to the NLD treatments, and no significant differences in stem Ca concentration were found. This indicates that considerable amounts of Ca may have been absorbed by the older, brown or woody roots as suggested before (Atkinson and Wilson, 1979, 1980; Comerford et al., 1994; Baldi et al., 2010). The use of electronic dendrometers to measure maximum daily stem shrinkage (MDS) demonstrated that sap flow rates in AD trees remained above zero during winter, suggesting that leaf transpiration-driven sap flow is not vital for root Ca uptake and translocation in the xylem during dormancy. The drivers responsible could not be established at present, but previous studies indicated that stem transpiration via natural openings (Groh et al., 2002; Pfanz et al., 2002) and positive root pressure under conditions of little or no transpiration (Tromp and Van Vuure, 1993; Tanner and Beevers, 2001; Wegner, 2014) could contribute to upward xylem sap flow and nutrient translocation in plants. In contrast to previous findings in apple (Van Zyl, 2016), in trees that dropped their leaves naturally, an increase in autumn soil Ca supply resulted in a significant increase in leaf Ca concentration at the end of winter following the extended leaf drop period. Root, stem and shoot Ca concentrations were, however, not affected. In agreement with others (Terblanche, 1972; Conradie, 1981; Stassen and Stadler, 1988;

Kangueehi et al., 2011), to the detriment of Ca allocation to the reserve tissues during winter, a substantial fraction (± 37 %) of total Ca uptake in the $2X_{(NLD)}$ treatment was lost via leaf abscission following transport to the leaves. Since the distribution of free Ca in the xylem sap is closely related to the intensity of transpiration (Clarkson, 1984; White and Broadley, 2003; Gilliham et al., 2011), an increase in Ca allocation to the leaves was possibly the result of relative high rates of leaf transpiration-driven sap flow prior to 50 % leaf drop in the $2X_{(NLD)}$ treatment as demonstrated by the MDS in these trees.

Thus, in areas with insufficient winter chilling such as Stellenbosch, an extended leaf drop period following synchronized soil Ca applications with active white root growth, can have a negative impact on Ca reserve accumulation in the roots and stems of young, non-bearing apple trees during autumn and into winter, but not on root growth.

Three weeks after the last soil Ca applications in May 2016, white root tips were harvested from the control and $2X_{(NLD)}$ treatment at bi-weekly to monthly intervals until the end of winter. Calcium was quantified in two separate locations (parenchyma cells in the mid-cortex and xylem vessels in the stele) at 5 mm (apical) and 20 mm (basal), respectively, from the apex of each root tip. No significant differences in root Ca concentration between the control and $2X_{(NLD)}$ treatment were found. Since the Ca concentration of the leaves of the $2X_{(NLD)}$ treatment was significantly higher compared to the control at the end of winter, active uptake of additional soil-applied Ca likely occurred along the entire length of apple white root tips during winter. The relative contributions of the apoplastic and symplastic pathways for Ca transport to the xylem could not be established with certainty, as scanning electron micrographs of white root cross-sections did not provide the necessary information on root endodermal, i.e. State I (Casparian band deposition) and II (suberin deposition), development (Mackenzie, 1979; White, 2001; Geldner, 2013). Based on previous findings (Kuhn et al., 2000; Cholewa and Peterson, 2004), results on Ca distribution between the cortex and stele did, however, suggest a differential pathway of Ca translocation to the shoot before and after 50 % leaf drop in July, as root tips harvested after 50 % leaf drop had a significant higher Ca concentration in the cortex compared with those harvested prior. As previously described (Wilcox, 1962; Ferguson and Clarkson, 1975; Waisel and Eshel, 2002; Enstone et al., 2003), state I and II development tends to occur further from the root tip in faster growing roots compared with slower growing roots. Consequently, in faster growing roots, nutrient uptake can occur via the apoplast at an increased distance from the tip. In contrast, in slower growing roots, state I and II development

closer to the tip dictates symplastic flow across the endodermis, the latter allowing roots to control the rate and selectivity of Ca transport to the shoot based on demand (Clarkson, 1984, 1993; White, 1998, 2001; Engels, 1999; White and Broadley, 2003; Wang et al., 2006). Our results, thus, suggest that Ca translocation to the shoot prior to 50 % leaf drop mainly proceeded via the apoplast in faster growing roots at a rate that matched transpirational demand, whereas Ca translocation to the shoot after 50 % leaf drop mainly proceeded via the symplast in slower growing roots at a rate that matched shoot demand.

In the third trial, $Ca(NO_3)_2$ was applied to the soil in summer (November 2015 and 2016) as a summer-only (2X) treatment, in autumn (May 2016) as an autumn-only (2X) treatment, and both in summer and autumn as a summer (1X)/autumn (1X) treatment during periods of active root growth over two seasons. No significant differences in fruit Ca concentration between treatments were evident at harvest, 120 days after full bloom (DAFB) in the second season. The lack of response could not be ascribed to untimely supply, as soil applications were synchronized with periods of active white root growth in both seasons. A possible explanation for the inefficiency of summer applications in increasing fruit Ca concentration at harvest, is that current season soil applications were applied after xylem dysfunction in the fruit had occurred (Dražeta et al., 2004, Amarante et al., 2013; Miqueloto et al., 2014; Le Roux, 2018). Since Ca^{2+} ions taken up by the roots are predominantly transported in the xylem with the transpiration stream (Hanger, 1979; Ferguson, 1980; White and Broadley, 2003), xylem vessel rupture and disintegration with fruit expansion from approximately 56 DAFB (Le Roux, 2018) possibly resulted in a reduced supply of Ca^{2+} to the fruit via root uptake. Whilst confirming previous findings of primary xylem transport to the more dominant leaf and shoot sinks at that time (Van Zyl, 2016), the lack of response to summer applications in November 2016 provides further evidence of the crucial role of Ca reserve accumulation in the roots and stems of apple trees in supporting fruit growth early in the following season (Terblanche, 1972; Terblanche et al., 1979) when new roots are mostly absent (Van Zyl, 2016) and the majority of Ca taken up by the leaves during rapid shoot growth cannot be redistributed to the fruit via the phloem (Mengel, 2002; White and Broadley, 2003; Hirschi, 2004; Gilliham et al., 2011).

Although the Ca reserve pool resulting from summer (1X and 2X, respectively) and autumn (1X) applications in the previous season failed to contribute substantially to fruit Ca content (g DM) in the following season, a significantly higher % of total Ca content was found in the fruit of the autumn-only (2X) treatment. Although a relatively high percentage of total Ca uptake

may have been lost via normal leaf abscission as a result of the extended leaf drop period, the remaining fraction of available Ca that was taken up by the roots and stored as reserves following relatively high $Ca(NO_3)_2$ soil applications at the beginning of the second root flush, in winter, seemed sufficient to increase fruit Ca content early in the following season. As this effect was not observed in the fruit of summer/autumn treatment, locally, a positive response to late-season $Ca(NO_3)_2$ soil applications seems to depend on the rate of application. Since mineral analyses also indicated a significant increase in root- and stem Ca concentration in the summer-only treatment at the end of April 2017, the current strategy of synchronizing high rates of additional soil Ca supply with periods of active white root growth in young, bearing 'Golden Delicious' apple trees, both in summer and autumn, may benefit the following season's crop via remobilization of stored Ca from the roots and reserve tissues of the stems. To confirm these findings in: (1) bitter pit-prone apple cultivars in the Western Cape and (2) bearing apple trees in the field, a longer trial period comprising high rates of soil Ca supply, both in summer and autumn during active white root growth, is advised.

In developing effective Ca fertilization strategies to optimize apple fruit quality in local orchards, assessing Ca uptake and distribution in apple trees following synchronized soil Ca applications with active white root growth is a fundamental step towards a better understanding of the role of Ca. Despite evidence of rapid Ca uptake by young white roots or root tips during autumn and into winter, the functional role of Ca uptake by older, brown roots in dormant, leafless apple trees remains to be elucidated. Although there are reports that brown and/or woody apple roots function in Ca absorption (Atkinson and Wilson, 1979, 1980), it is uncertain whether the observed Ca uptake patterns in these studies were justified on an anatomical scale in relation to above-ground tree phenology. More research is therefore needed to further compare these different root categories by direct measures of root Ca uptake and translocation in relation to tree phenology and internal root developmental stages. As described by Cocozza et al. (2008), Hunsche and Noga (2008) and Tuladhar and Nii (2014), this can be achieved by applying a combination of investigative techniques, such as SEM-WDS and energy-dispersive x-ray spectroscopy (EDS), light microscopy following histochemical staining of lignin and suberin, and/or fluorescence microscopy with or without staining.

In addition to the effects of root age on Ca uptake and translocation in apple, differences in physiological function among individual roots within the fine root architecture of trees are also strongly related to their branching position or order (Pregitzer et al., 2002; Wells and Eissenstat,

2003; Hishi, 2007; McCormack et al., 2015). To match root functional types as closely as possible, it is further advised to adopt root sampling protocols that account for both spatial and temporal variation in root function (Freschet and Roumet, 2017; McCormack et al., 2017). Measurements of fine root traits (e.g. morphology, anatomy, physiology and mycorrhizal associations) would also enable comparable linkages to be made between fine root function and root trait variation in apple (Wells and Eissenstat, 2003; Hishi, 2007; McCormack et al., 2015; Lalibert, 2017). Root sampling should commence shortly after treatment, as soil Ca may become depleted in less than 14 days due to the high uptake and turnover rates of young, primary apple roots (Bouma et al., 2001). Considering the current method of split applications of $Ca(NO_3)_2$ to the soil, it is recommended that root sampling take place after each consecutive application in $\leq$ 24-hour increments after treatment. Root calcium uptake should also be quantified in different soil types under field conditions, as Ca uptake ability is higher for roots produced in sandy soil compared with clay and organic soils (Fan and Yang, 2011).

As the mechanism for sustained sap flow in leafless apple trees during winter could not be established, future studies could benefit by including additional measures of root pressure and stem transpiration as part of their investigations. Physiological measurements such as photosynthesis, carbon allocation and leaf transpiration are also advised. Finally, measurements of root growth rate in relation to above-ground tree phenology could also provide important information on Ca uptake and translocation processes along the length of apple roots, not only during winter dormancy, but also during the rest of the growing season.

To conclude, in young, potted, ‘Golden Delicious’/M7 apple trees established in an area with insufficient winter chilling, an increased supply of Ca from high rates of $Ca(NO_3)_2$ fertilizer applied to the soil at the beginning of the second root flush, in winter, contributed significantly towards the Ca content of the following season’s fruit after only one season. Although Ca was primarily allocated to the leaves, and not the reserves following an extended leaf drop period, the remaining reserves, as accumulated in the roots and stems during late-autumn and into winter, seemed sufficient to support fruit growth early in the following season. The same effect was not observed in the fruit of the summer/autumn treatment that received a relatively low supply of Ca in autumn. Likewise, the application of high rates of $Ca(NO_3)_2$ fertilizer to the soil at the beginning of the first root flush, in summer, failed to significantly increase fruit Ca content at harvest after two consecutive seasons. However, as summer applications caused a significant increase in root and stem Ca concentration in the second season, preharvest soil Ca

supply would therefore be regarded primarily as a means to increase the Ca reserve status of the tree. Thus, in locally-grown ‘Golden Delicious’ apple trees, routine applications of high rates of $Ca(NO_3)_2$ fertilizer to the soil during periods of active white root growth in summer and autumn are recommended to replenish and maintain Ca reserves in the roots and woody tissues of the tree for early-season transfer to the following season’s crop, when direct supply of soil-applied Ca from the roots is still inadequate. Furthermore, since autumn defoliation prior to high rates of soil $Ca(NO_3)_2$ supply favoured Ca allocation to the roots and reserve tissues of locally-grown ‘Golden Delicious’ apple trees during winter, a lack of knowledge on the effects of such a practice on fruit Ca concentration of the following season’s crop warrants further investigation.

References

Amarante, C.V.T., Miqueloto, A., Steffens, C.A., dos Santos, A., Argenta, L.C., 2013. Changes in xylem functionality during apple fruit development: Implications on calcium concentration and incidence of bitter pit. Acta Hort. 1012, 135–140.

Atkinson, D., Wilson, S.A., 1979. The root-soil interface and its significance for fruit tree roots of different ages, in: Harley, J.L., Russel, R.S. (Eds.), The Soil-Root Interface. Academic Press, London, pp. 259–271.

Atkinson, D., Wilson, S.A., 1980. The growth and distribution of fruit tree roots: Some consequences for nutrient uptake, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 137–150.

Baldi, E., Wells, C.E., Marangoni, B., 2010. Nitrogen absorption and respiration in white and brown peach roots. J. Plant Nutr. 33, 461–469.

Bouma, T.J., Yanai, R.D., Elkin, A.D., Hartmond, U., Flores-Alva, D.E., Eissenstat, D.M., 2001. Estimating age-dependent costs and benefits of roots with contrasting life span: Comparing apples and oranges. New Phytol. 150, 685–695.

Cholewa, E., Peterson, C.A., 2004. Evidence for symplastic involvement in the radial movement of calcium in onion roots. Plant Physiol. 134, 1793–1802.

Clarkson, D.T., 1984. Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. 7, 449–456.

Clarkson, D.T., 1993. Roots and the delivery of solutes to the xylem. Philos. Trans. R. Soc. Biol. Sci. 341, 5–17.

Cocozza, C., Minnocci, A., Tognetti, R., Iori, V., Zacchini, M., Scarascia Mugnozza, G., 2008. Distribution and concentration of cadmium in root tissue of *Populus alba* determined by scanning electron microscopy and energy-dispersive x-ray microanalysis. IForest 1, 96–103.

Comerford, N.B., Smethurst, P.J., Escamilla, J.A., 1994. Nutrient uptake by woody root systems. New Zeal. J. For. Sci. 24, 195–212.

Conradie, W.J., 1981. Seasonal uptake of nutrients by Chenin blanc in sand culture: II. Phosphorus, potassium, calcium and magnesium. South African J. Enol. Vitic. 2, 7–13.

Dražeta, L., Lang, A., Hall, A.J., Volz, R.K., Jameson, P.E., 2004. Causes and effects of changes in xylem functionality in apple fruit. Ann. Bot. 93, 375–382.

Emanuelsson, J., 1984. Root growth and calcium uptake in relation to calcium concentration. Plant Soil 78, 325–334.

Engels, C., 1999. Regulation of xylem transport of calcium from roots to shoot of maize by growth-related demand. J. Plant Nutr. Soil Sci. 162, 287–294.

Enstone, D.E., Peterson, C.A., Ma, F., 2003. Root endodermis and exodermis: Structure, function, and responses to the environment. Plant Growth Regul. 21, 335–351.

Fan, W., Yang, H., 2011. Effect of soil type on root architecture and nutrient uptake by roots of young apple rootstocks. Acta Hort. 903, 885–890.

Ferguson, I.B., 1980. The uptake and transport of calcium in the fruit tree, in: Atkinson, D., Jackson, J.E., Sharples, R.O., Waller, W.M. (Eds.), Mineral Nutrition of Fruit Trees. Butterworths, London-Boston, pp. 183–192.

Ferguson, I.B., Clarkson, D.T., 1975. Ion transport and endodermal suberization in the roots of *Zea mays*. New Phytol. 75, 69–79.

Freschet, G.T., Roumet, C., 2017. Sampling roots to capture plant and soil functions. Funct. Ecol. 31, 1506–1518.

Geldner, N., 2013. The endodermis. Annu. Rev. Plant Biol. 64, 531–558.

Gilliham, M., Dayod, M., Hocking, B.J., Xu, B., Conn, S.J., Kaiser, B.N., Leigh, R.A., Tyerman, S.D., 2011. Calcium delivery and storage in plant leaves: Exploring the link with water flow. J. Exp. Bot. 62, 2233–2250.

Groh, B., Hübner, C., Lendzian, K.J., 2002. Water and oxygen permeance of phellems isolated from trees: The role of waxes and lenticels. Planta 215, 794–801.

Hanger, B.C., 1979. The movement of calcium in plants. Commun. Soil Sci. Plant Anal. 10, 171–193.

Head, G.C., 1969. The effects of fruiting and defoliation on seasonal trends in new root production on apple trees. J. Hort. Sci. 44, 175–181.

Hewett, E.W., 2006. An overview of preharvest factors influencing postharvest quality of horticultural products. Int. J. Postharvest Technol. Innov. 1, 4–15.

Hirschi, K.D., 2004. The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol. 136, 2438–2442.

Hishi, T., 2007. Heterogeneity of individual roots within the fine root architecture: Causal links between physiological and ecosystem functions. J. For. Res. 12, 126–133.

Hunsche, M., Noga, G., 2008. Applicability of the energy dispersive x-ray microanalysis for quantification of irregular calcium deposits on fruit and leaf cuticles. J. Microsc. 232, 453–462.

Jemrić, T., Fruk, I., Fruk, M., Radman, S., Sinkovič, L., Fruk, G., 2016. Bitter pit in apples: Pre- and postharvest factors: A review. Spanish J. Agric. Res. 14, 1–12.

Kangueehi, G.N., Stassen, P.J.C., Theron, K.I., Wooldridge, J., 2011. Macro and micro element requirements of young and bearing apple trees under drip fertigation. South African J. Plant Soil 28, 136–141.

Kuhn, A.J., Schröder, W.H., Bauch, J., 2000. The kinetics of calcium and magnesium entry into mycorrhizal spruce roots. Planta 210, 488–496.

Lalibert, E., 2017. Below-ground frontiers in trait-based plant ecology. New Phytol. 213, 1597–1603.

Le Roux, E., 2018. Ca-metalosate as an alternative Ca formulation for decreasing Ca related disorders in fruit trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Lötze, E., Joubert, J., Theron, K.I., 2008. Evaluating pre-harvest foliar calcium applications to increase fruit calcium and reduce bitter pit in 'Golden Delicious' apples. Sci. Hort. 116, 299–304.

Lötze, E., Theron, K.I., 2006. Dynamics of calcium uptake with pre-harvest sprays to reduce bitter pit in 'Golden Delicious'. Acta Hort. 721, 313–319.

Lötze, E., Theron, K.I., 2007. Evaluating the effectiveness of pre-harvest calcium applications for bitter pit control in 'Golden Delicious' apples under South African conditions. J. Plant Nutr. 30, 471–485.

Mackenzie, K.A.D., 1979. The development of the endodermis and phi layer of apple roots. Protoplasma 100, 21–32.

Maggs, D.H., 1965. Growth rates in relation to assimilate supply and demand: II. The effect of particular leaves and growing regions in determining the dry matter distribution in young

apple trees. J. Exp. Bot. 16, 387–404.

McCormack, M.L., Dickie, I.A., Eissenstat, D.M., Fahey, T.J., Fernandez, C.W., Guo, D., Helmisaari, H., Hobbie, E.A., Iversen, C.M., Jackson, R.B., Leppalammi-Kujansuu, J., Norby, R.J., Phillips, R.P., Pregitzer, K.S., Pritchard, S.G., Rewald, B., Zadworny, M., 2015. Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes. New Phytol. 207, 505–518.

McCormack, M.L., Guo, D., Iversen, C.M., Chen, W., Eissenstat, D.M., Fernandez, C.W., Li, L., Ma, C., Ma, Z., Poorter, H., Reich, P.B., Zadworny, M., Zanne, A., 2017. Building a better foundation: Improving root-trait measurements to understand and model plant and ecosystem processes. New Phytol. 215, 27–37.

Mengel, K., 2002. Alternative or complementary role of foliar supply in mineral nutrition. Acta Hort. 594, 33–47.

Miqueloto, A., Amarante, C.V.T., Steffens, C.A., dos Santos, A., Mitcham, E., 2014. Relationship between xylem functionality, calcium content and the incidence of bitter pit in apple fruit. Sci. Hort. 165, 319–323.

Nightingale, G.T., 1935. Effects of temperature on growth, anatomy, and metabolism of apple and peach roots. Bot. Gaz. 96, 581–639.

Pfanz, H., Aschan, G., Langenfeld-Heyser, R., Wittmann, C., Loose, M., 2002. Ecology and ecophysiology of tree stems: Corticular and wood photosynthesis. Naturwissenschaften 89, 147–162.

Poovaiah, B.W., Reddy, A.N., 1991. Calcium and root development: Importance of calcium in signal transduction, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 205–227.

Pregitzer, K.S., Deforest, J.L., Burton, A.J., Allen, M.F., Ruess, W.R., Hendrick, R.L., 2002. Fine root architecture of nine North American trees. Ecol. Monogr. 72, 293–309.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: Its mechanism and endogenous control. Sci. Hort. 105, 65–89.

Stassen, P.J.C., Stadler, J.D., 1988. Seasonal uptake of phosphorus, potassium, calcium and magnesium by young peach trees. South African J. Plant Soil 5, 19–23.

Tanner, W., Beevers, H., 2001. Transpiration, a prerequisite for long-distance transport of

minerals in plants? Proc. Natl. Acad. Sci. 98, 9443–9447.

Terblanche, J.H., 1972. Seisoensopname en verspreiding van tien voedingselemente by jong appelbome gekweek in sandkulture. Ph.D. dissertation, Faculty of Agricultural Science, Stellenbosch University.

Terblanche, J.H., Wooldridge, L.G., Hesebeck, I., Joubert, M., 1979. The redistribution and immobilisation of calcium in apple trees with special reference to bitter pit. Commun. Soil Sci. Plant Anal. 10, 195–215.

Tromp, J., Van Vuure, J., 1993. Accumulation of calcium, potassium and magnesium in apple fruits under various conditions of humidity. Physiol. Plant. 89, 149–156.

Tuladhar, A., Nii, N., 2014. An anatomical study of developmental changes in maturing root tissues of pomegranate (*Punica granatum* L.) and formation of a unique type of periderm. Plant Root 8, 33–41.

Van Zyl, F.J., 2016. Quantifying root growth dynamics and nutrient uptake in apple trees. MSc thesis, Faculty of Agrisciences, Stellenbosch University.

Waisel, Y., Eshel, A., 2002. Functional diversity of various constituents of a single root sytem, in: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: The Hidden Half. Marcel Dekker, Inc., New York, NY, USA, pp. 157–174.

Wang, H., Inukai, Y., Yamauchi, A., 2006. Root development and nutrient uptake. Crit. Rev. Plant Sci. 25, 279–301.

Wegner, L.H., 2014. Root pressure and beyond: Energetically uphill water transport into xylem vessels? J. Exp. Bot. 65, 381–393.

Wells, C.E., Eissenstat, D.M., 2003. Beyond the roots of young seedlings: The influence of age and order on fine root physiology. J. Plant Growth Regul. 21, 324–334.

White, P.J., 1998. Calcium channels in the plasma membrane of root cells. Ann. Bot. 81, 173–183.

White, P.J., 2001. The pathways of calcium movement to the xylem. J. Exp. Bot. 52, 891–899.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilcox, H., 1962. Growth studies of the root of incense cedar, *Libocedrus decurrens*. I. The origin and development of primary tissues. Am. J. Bot. 49, 221–236.

Zhang, H., Rong, H., Pilbeam, D., 2007. Signalling mechanisms underlying the morphological responses of the root system to nitrogen in *Arabidopsis thaliana*. J. Exp. Bot. 58, 2329–2338.

ANNEXURE 1

Table 1. Mineral analysis of the potting medium (4:1 *v*/*v* mixture of sand and compost) at planting (September 2015) prior to any treatments.

pH (KCl)	P Olsen (mg/kg)	P Bray II (mg/kg)	Na (cmol/kg)	K (cmol/kg)	Ca (cmol/kg)	Mg (cmol/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Fe (mg/kg)	C %	N %
7.4	16.0	52.0	0.6	0.6	5.2	1.0	0.6	4.7	4.2	0.4	43.0	1.1	0.05

Table 2. Calcium [a] and nitrogen [b] content (g DM), respectively, in the roots, stems (two-year-old wood), shoots (one-year-old wood including spurs) and leaves of two-year-old, potted, non-bearing 'Golden Delicious'/M7 apple trees at the end of August 2016 in response to $Ca(NO_3)_2$ soil applications in May 2016.

Treatment	Calcium (g DM)				Nitrogen (g DM)			
	Roots	Stems	Shoots	Leaves [f]	Roots	Stems	Shoots	Leaves [f]
Natural leaf drop (NLD)								
Control (None)	0.37 ns	0.65 ns	0.24 ns	---	1.57 b	1.85 ab	0.45 a	---
1X (Moderate) [c]	0.34	0.59	0.21	---	2.29 a	2.21 a	0.65 a	---
2X (High) [d]	0.27	0.40	0.19	0.53 a	2.19 a	1.91 ab	0.57 a	0.61 ns
Autumn defoliation (AD)								
2X (High) [e]	0.27	0.43	0.07	0.35 b	1.11 b	1.20 b	0.14 b	0.75
P-value	0.2240	0.0856	0.0102	0.0090	< 0.0001	0.0127	< 0.0001	0.0701

	Total Ca (g DM) per tree [g]	Total N (g DM) per tree [g]
2X (NLD) [d]	1.44 ns	5.28 a
2X (AD) [e]	1.15	3.20 b
P-value	0.1327	0.0032

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] Ca content (g DM) in each plant part = (Ca concentration (% DM) of each plant part (Paper 1, Table 3)) × (Corresponding DM (g) of each plant part (Table 2)).

[b] N content (g DM) in each plant part = (N concentration (% DM) of each plant part (Paper 1, Table 3)) × (Corresponding DM (g) of each plant part (Table 2)).

[c] 75 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[d] 150 g $Ca(NO_3)_2$ pot^{-1} prior to the onset of leaf drop mid-May 2016.

[e] 150 g $Ca(NO_3)_2$ pot^{-1} after leaf removal in April 2016.

[f] Because most of the leaves of the control and 1X treatment were lost during natural leaf drop, the Ca and N content (g DM) in the leaves as well as the total Ca and N content per tree in these two treatments were not calculated.

ANNEXURE 2

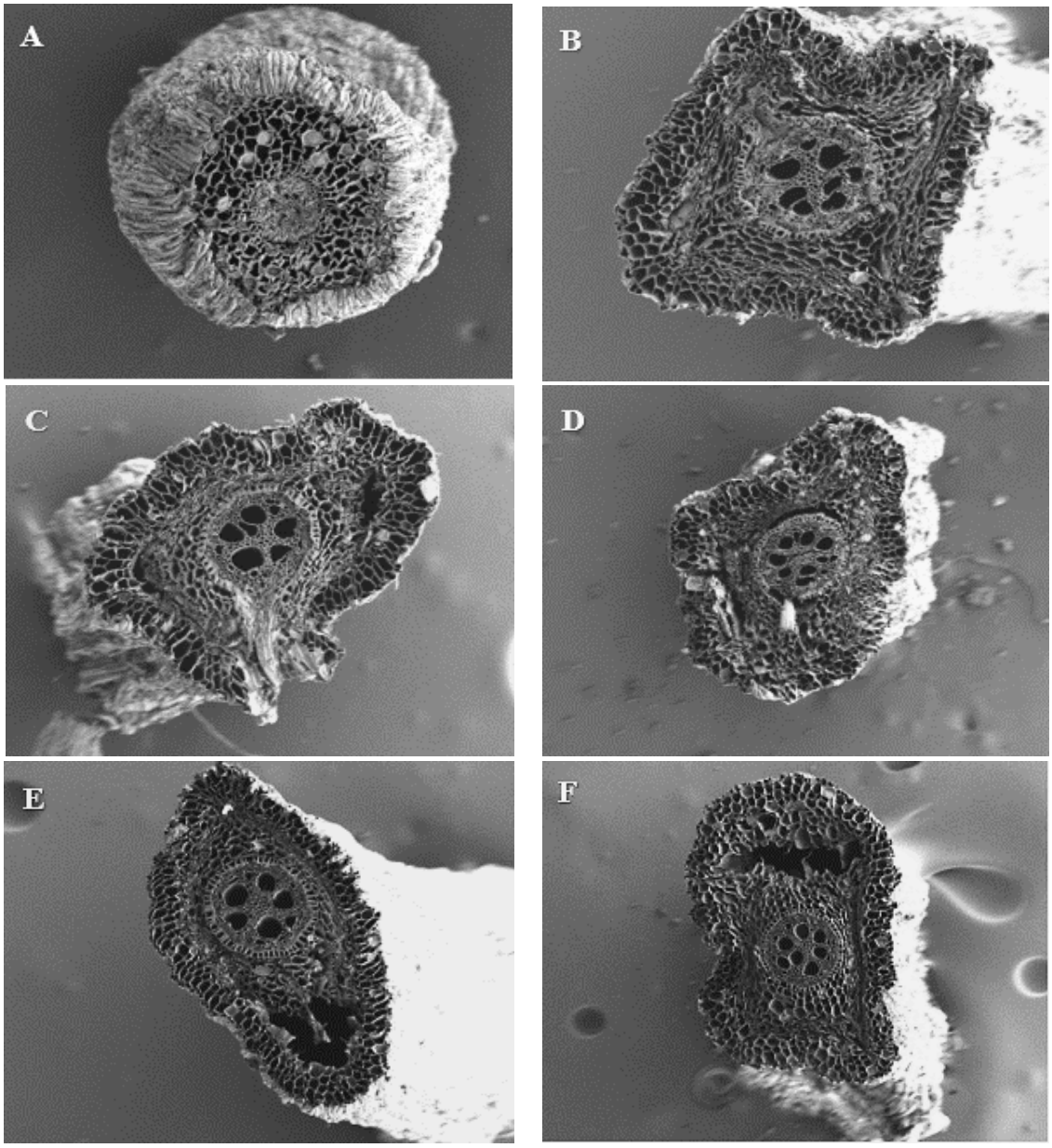


Fig. 1. Digital images taken with a scanning electron microscope (Carl Zeiss SMT Ltd. MERLIN Field Emission Gun, ZEISS, Germany) of white root segments that underwent fixation (GA)/post-fixation (OsO_4), dehydration (ascending ethanol series) and drying (CPD) (A); fixation (GA)/post-fixation (OsO_4), dehydration (ascending ethanol series) and drying (HMDS) (B); fixation (modified Karnovsky's fixative)/post-fixation (OsO_4), dehydration (ascending ethanol series) and drying (CPD) (C); fixation (modified Karnovsky's fixative)/post-fixation (OsO_4), dehydration (ascending ethanol series) and drying (HMDS) (D); fixation (GA), dehydration (ascending ethanol series) and drying (CPD) (E); fixation (GA), dehydration (ascending ethanol series) and drying (HMDS) (F).

ANNEXURE 3

Table 1: Calcium content (g DM) [a] in the roots, stems (two- and three-year-old wood), new growth (one-year-old shoots including spurs and leaves) and fruit (stalks, core and pips removed) of three-year-old, potted, 'Golden Delicious'/M7 apple trees in response to different timings of $Ca(NO_3)_2$ soil applications. Fruit was harvested at the end of January 2017 (120 DAFB). Trees were destructively harvested at the end of April 2017.

Treatment	Roots	Stems	New growth	Fruit
Control	1.42 ab	2.25 b	2.00 ns	0.06 ns
Summer [b]	1.73 a	3.63 a	2.51	0.07
Autumn [c]	1.14 b	2.26 b	1.80	0.10
Summer/Autumn [d]	1.77 a	2.87 ab	2.70	0.09
P-value	0.0268	0.0375	0.1319	0.1251

	Total Ca (g DM) per tree
Control	5.73 bc
Summer [b]	7.95 a
Autumn [c]	5.38 c
Summer/Autumn [d]	7.43 ab
P-value	0.0263

Means with the same letters are not significantly different at $P \leq 0.05$.

[a] Ca content (g DM) in each plant part = (Ca concentration (% DM) of each plant part (Paper 3, Table 3)) × (corresponding DM (g) of each plant part (Paper 3, Table 2)).

[b] 150 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16.

[c] 150 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

[d] 75 g $Ca(NO_3)_2$ pot^{-1} in November 2015/16; 75 g $Ca(NO_3)_2$ pot^{-1} in May 2016.

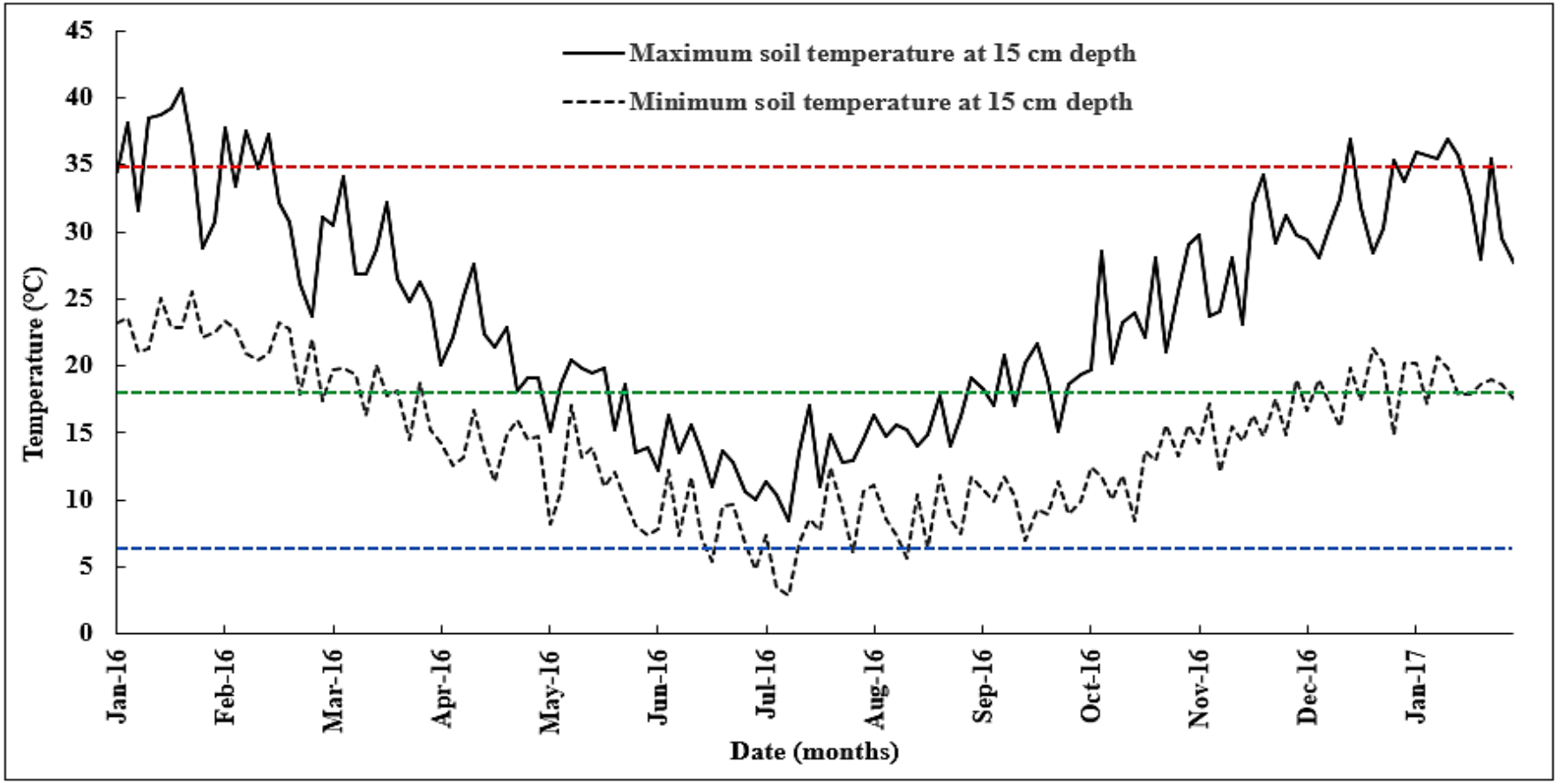


Fig. 1. Average soil temperatures of two randomly selected pots, filled with a 4:1 (*v*/*v*) mixture of coarse sand and compost, at a depth of 15 cm. Temperatures were recorded on the hour with Tiny-tag data logger soil probes (TPG-4505 Gemini Data Loggers Ltd., Chichester, West Sussex, UK) for the period January 2016 to January 2017. The green line depicts the optimum root growth temperature, the red line, the maximum temperature for root growth, and the blue line, the minimum temperature for root growth (Nightingale, 1935).